12-2002

Role of Dietary Calcium in Modulation of Adiposity

Xuemei Geng
University of Tennessee, Martin

To the Graduate Council:

I am submitting herewith a thesis written by Xuemei Geng entitled "Role of Dietary Calcium in Modulation of Adiposity." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Michael B. Zemel, Major Professor

We have read this thesis and recommend its acceptance:

Jean Skinner, Jung Han Kim

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Xuemei Geng entitled " Role of dietary calcium in modulation of adiposity." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Michael B. Zemel, Ph.D., Major Professor

We have read this thesis and
recommend its acceptance:

Jean Skinner, Ph.D.

Jung Han Kim, Ph.D.

Accepted for the Council:

Vice Provost and
Dean of Graduate Studies

ROLE OF DIETARY CALCIUM IN MODULATION OF ADIPOSITY

A Thesis
Presented for the
Master of Science in Nutrition
Degree
The University of Tennessee, Knoxville

Xuemei Geng
December 2002

DEDICATION

This thesis is dedicated to my parents, Mr. Tianyun Geng and Mrs. Shufang Wang, to my husband Bing Yu and our lovely daughter Angela Yu. Their enormous love and support enabled me to complete this thesis.

ACKNOWLEDGMENTS

I would like to thank all those who helped me in completing Master of Science in Nutrition. I thank my committee members, Dr. Michael B. Zemel, Dr. Jean Skinner and Dr. Jung Han Kim for their constructive advice. I am especially grateful to my advisor, Dr. Michael B. Zemel for his continued encouragement and support throughout my graduate studies. I would also like to thank Hang Shi, a former graduate student in Nutrition Department now a postdoc at Harvard, for his help in the lab.

Finally I want to express my appreciation for my family and friends. Without their endless love and support through bad times and good, I could not reach this moment.

ABSTRACT

We previously demonstrated a regulatory role for intracellular Ca^{2+} ($[Ca^{2+}]_i$) in human and murine adipocyte metabolism, with increased $[Ca^{2+}]_i$ coordinately stimulating lipogenesis and inhibiting lipolysis, thereby expanding adipocyte lipid mass. More recently, we reported that 1α, 25-dihydroxyvitamin D_3 [1α, 25-$(OH)_2$-D_3] stimulates adipocyte membrane vitamin D receptor (mVDR)-mediated Ca^{2+} influx, resulting in a similar Ca^{2+}-mediated modulation of adipocyte lipid metabolism. 1α, 25-$(OH)_2$-D_3 also exerts an inhibitory effect on adipocyte uncoupling protein 2 (UCP2) mRNA and protein levels via the nuclear vitamin D receptor (nVDR), independent of its effects on Ca^{2+} influx. Furthermore, we reported that suppressing 1α, 25-$(OH)_2$-D_3 levels by increasing dietary calcium reduces adipocyte $[Ca^{2+}]_i$, stimulates lipolysis, inhibits lipogenesis, increases thermogenesis, and consequently results in attenuation of adipocyte lipid accretion and weight/fat gain during over-consumption and acceleration of weight/fat loss during energy restriction. Notably, dairy sources of calcium exerted significantly greater effects than calcium in the form of calcium carbonate, although the reason for this is not yet clear. This thesis study was conducted to extend these observations by determining the efficacy of a fermented dairy product, yogurt, presented either unflavored or strawberry-flavored, compared to calcium carbonate, in accelerating weight/fat loss secondary to caloric restriction in aP2-agouti transgenic mice. Mice were fed a low calcium (0.4%)/high fat/high sucrose diet for 6 weeks in order to induce obesity. Mice were then either maintained on the same low calcium basal diet *ad lib* or energy-

restricted (70% of *ad lib*) on this diet either unsupplemented (basal) or supplemented with calcium content increased to 1.2% either by replacing 34% of the protein with spray dried plain or strawberry-flavored yogurt (with macronutrient adjustments) or by adding calcium carbonate for 6 weeks. Adipocyte $[Ca^{2+}]_i$ was only slightly decreased by energy restriction alone but was further markedly reduced by all three high calcium diets (170±6 nM, n=10 vs. 60±5 nM, n=30, $p<0.001$). The three high calcium diets caused significant stimulation of both basal and isoproterenol-stimulated lipolysis (78%-137%, $p<0.05$), with yogurt exerting a significantly greater effect compared to calcium carbonate ($p<0.05$). Similarly, the three high calcium diets suppressed adipocyte FAS mRNA by an average of 87%. Body weight, as well as individual and total fat pad weights, reflected these changes, with a 45% augmentation in fat loss on the two yogurt diets compared to that on the low calcium diet secondary to caloric restriction ($p<0.001$). Calcium carbonate supplementation produced an intermediate effect, with a lower 28% augmentation in fat loss compared to that on the low calcium diet secondary to caloric restriction. Furthermore, high calcium diets caused a higher core temperature and an increased white adipose tissue UCP2 expression, indicating increased thermogenesis and decreased metabolic efficiency. In conclusion, our data show that dietary calcium with calcium sources from plain yogurt, strawberry-flavored yogurt and calcium carbonate supplement suppress adipocyte $[Ca^{2+}]_i$ and up-regulate UCP2 via suppression of 1α, 25-$(OH)_2$-D_3, and thereby reduce energy storage, decrease metabolic efficiency and accelerate weight/ fat loss during energy restriction, with significantly greater effects exerted by yogurt than by

supplemental calcium. Consistent with the animal observations, accumulating human studies strongly support an anti-obesity role for dietary calcium. Therefore, dietary calcium, especially dairy calcium, may be effective dietary regimen for the treatment of obesity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURES PAGE

LIST OF ABBREVIATIONS

ACTH: adrenocorticotropic hormone

aP2: adipose tissue-specific fatty acid binding protein

ACE: angiotensin-I converting enzyme

AII: angiotensin II

β-ARs: β-adrenergic receptors

BAT/WAT: brown/white adipose tissue

CPE: carboxypeptidase E

CRH: corticotropin-releasing hormone

DIT: diet-induced thermogenesis

1α, 25-$(OH)_2$-D_3: 1α, 25-dihydroxy vitamin D_3

FAS: fatty acid synthase

GCR: glucocorticoid receptor

HPA: hypothalamic-pituitary-adrenal axis

$[Ca^{2+}]_i$: intracellular Ca^{2+} concentration

α-MSH: α-melanocyte-stimulating hormone

MC4-R: melanocortin-4 receptor

mVDR/nVDR: membrane/nuclear vitamin D receptor

POMC: pro-opiomelanocortin

ROS: reactive oxygen species

SNS: sympathetic nervous system

UCP: Uncoupling protein

PART 1

INTRODUCTION

INTRODUCTION

Obesity has reached epidemic proportions in the United States and is threatening to become a global epidemic (1). Overeating and physical inactivity in combination with genetic factors play the most important roles in the development of obesity (2). Since obesity is strongly associated with an increased incidence of chronic diseases (3), weight loss itself has been proven to be beneficial to reduce the morbidity and mortality risk associated with obesity (4-6). Americans spend about $40 billion per year on weight-loss treatments, mostly in the form of diets and dietary foods. And this approach seems to be ineffective (7).

Since McCarron first noted a significant inverse relationship between dietary calcium intake and body weight in his NHANES I data analysis in 1984 (8), accumulating body of evidence strongly support the anti-obesity role of dietary calcium (9-22). However, it is not until recently that the mechanism underlying this dietary calcium modulation of adiposity is explored.

Previous studies of the mechanism of action of the agouti gene in obesity and insulin resistance from our laboratory demonstrated a regulatory role for intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in human and murine adipocyte lipid metabolism (23-26). Increased adipocyte $[Ca^{2+}]_i$ appears to promote expansion of adipocyte triglyceride stores by exerting a coordinated stimulation of lipogenesis and inhibition of lipolysis. Moreover, obesity in transgenic mice ubiquitously expressing agouti under the control of the β-actin promoter was markedly attenuated by 4-week treatment of a Ca^{2+}- channel antagonist (nifedipine) (27).

More recently, we reported that 1α,25-$(OH)_2$-D_3 stimulates adipocyte Ca^{2+} influx via the mVDR, resulting in a similar Ca^{2+}-mediated modulation of adipocyte lipid metabolism (13, 28). 1α, 25-$(OH)_2$-D_3 also exerts an inhibitory effect on adipocyte UCP2 mRNA and protein levels via the nVDR, independent of its effects on Ca^{2+} influx (29). Furthermore, we reported that feeding high calcium diets (non-fat dry milk) results in decreased adipocyte $[Ca^{2+}]_i$, increased thermogenesis, and attenuation of adipocyte lipid accretion and weight/fat gain in *ad libitum* fed mice and marked acceleration of weight/fat loss secondary to caloric restriction (13, 16, 17). Accordingly, we proposed that suppressing 1α,25-$(OH)_2$-D_3 levels by increasing dietary calcium would suppress adipocyte $[Ca^{2+}]_i$, up-regulate UCP2, and consequently inhibit lipogenesis, stimulate lipolysis, increase thermogenesis and decrease metabolic efficiency, thereby exerting an anti-obesity effect.

Consistent with the animal observations, clinical and epidemiological observations support these findings (13, 15, 21). Epidemiologically, the effect of calcium intake on fat mass in humans was examined in the NHANES III data set (13). After controlling for energy intake and energy expenditure, a strong inverse relationship was noted between calcium intake and body fat in both women and men. The odds ratio of being in the highest quartile of body fat was markedly reduced from 1.00 for the first quartile of calcium intake to 0.75, 0.40, and 0.16 for the second, third, and fourth quartiles of calcium intake, respectively.

Collectively, dietary calcium may exert its anti-obesity effect for humans through the mechanism we proposed above, and further investigation is needed to confirm this. Notably, dairy sources of calcium exerted substantially greater effects in acceleration of

weight/loss secondary to caloric restriction compared to the same levels of calcium in the form of calcium carbonate supplement (13, 16, 17) although the additional component(s) of dairy products responsible for this difference is not yet known. Moreover, the only dairy product systematically tested in this manner has been non-fat dry milk (13, 16), and it is not clear whether other forms of dairy products will exert comparable or greater weight-loss effects secondary to caloric restriction. Accordingly, the objective of this thesis research is to determine the efficacy of a fermented dairy product, yogurt, presented either unflavored or strawberry-flavored, compared to calcium carbonate, in acceleration of weight/fat loss secondary to caloric restriction in aP2-agouti transgenic mice.

LITERATURE CITED

1. World Health Organization (1998) *Obesity: Preventing and Managing the Global Epidemic* (World Health Organization, Geneva)

2. Arner, P. (2000) Hunting for human obesity genes? Look in the adipose tissue! *Intl. J. Obes.* **24**, Suppl. 4, S57-S62

3. World Health Organization (2000) Obesity: preventing and managing the global epidemic. Report of a WHO consultation. *World Health Organ. Tech. Rep. Ser.* **894**, i-xii, 1-253

4. Goldstein, D. J. (1992) Beneficial effects of modest weight loss. *Intl. J. Obes.* **16**, 397-415

5. Blackburn, G. (1995) Effect of degree of weight loss on health benefits. *Obes. Res.* **3** (Suppl. 2), 211S-218S

6. Williamson, D. F. (1995) Prospective study of intentional weight loss and mortality in never-smoking overweight US white women aged 40-64 years. *Am. J. Epidem.* **141**, 1128-1141

7. Wickelgren, I. (1998) Obesity: How big a problem? *Science* **280**, 1364-1367

8. McCarron, D. A., Morris, C. D., Henry, H. J., and Stanton, J. L. (1984) Blood pressure and nutrient intake in the United States. *Science* **224**, 1392-1398

9. Metz, J. A., Karanja, N., Torok, J., and McCarron, D. A. (1988) Modification of total body fat in spontaneously hypertensive rats and Wistar-Kyoto rats by dietary calcium and sodium. *Am. J. Hypertens.* **1**, 58-60

10. Bursey, R. D., Sharkey, T., and Miller, G. D. (1989) High calcium intake lowers weight in lean and fatty Zucker rats. *FASEB J.* **3**, A265 (Abstr.)

11. Fleming, K. H., and Heimbach, J. T. (1994): Consumption of calcium in the U. S.: Food sources and intake levels. *J. Nutr.* **124**, 1426S-1430S

12. Summerbell, C. D., Watts, C., Higgins, J. P. T., and Garrow, J. S. (1998) Randomized controlled trial of novel, simple, and well supervised weight reducing diets in outpatients. *Br. Med. J.* **317**, 1487-1489

13. Zemel, M. B., Shi, H., Greer, B., Dirienzo, D., and Zemel, P. C. (2000) Regulation of adiposity by dietary calcium. *FASEB J.* **14**, 1132-1138

14. Lin, Y., Lyle, R.M., McCabe, L.D., McCabe, G.P., Weaver, C.M., and Teegarden, D. (2000) Dairy calcium is related to changes in body composition during a two-year exercise intervention in young women. *J. Am. Col. Nutr.* **19**, 754-760

15. Davies, K.M., Heaney, R.P., Recker, R.R., Lappe, J.M., Barger-Lux, M.J., Rafferty K., and Hinders, S. (2000) Calcium intake and body weight. *J. Clin. Endocrinol. Metab.* **85**: 4635-4638

16. Shi, H., DiRienzo, D., and Zemel, M. B. (2001) Effects of dietary calcium on adipocyte lipid metabolism and body weight regulation in energy-restricted aP2-agouti transgenic mice. *FASEB J.* **15**, 291-293

17. Zemel, M. B., Sun, X., and Geng, X. (2001) Effects of calcium-fortified breakfast cereal on adiposity in a transgenic mouse model of obesity. *FASEB J.* **15**, A598 (Abstr.480.7)

18. Jacqmain, M., Doucet, E., Despres, J-P., Bouchard, C., and Tremblay, A. (2001) Calcium intake and body composition in adults. *Obes. Res.* **9** (Suppl.), 175S.PF104

19. Carruth, B.R., Skinner, J.D. (2001) The role of dietary calcium and other nutrients in moderating body fat in preschool children. *Int. J. Obes.* **25**, 559-566

20. Chan, G. M., and McNaught, T. P. (2002) Dietary calcium influences body fat in children. *Pedia. Res.* **51** (4), Part 2 Suppl., 202A.1173

21. Zemel, M. B., Thompson, W., Zemel, P., Nocton, A. M., Milstead, A., Morris, K., and Campbell, P. (2002) Dietary calcium and dairy products accelerates weight and fat loss during energy restriction in obese adults. *Am. J. Clin. Nutr.* **75** (2)(Suppl.), 342S.13

22. Pereira, M. A., Jacobs, D. R., Horn, L. V., Slattery, M. L., Kartashov, A. I., and Ludwig, D. S. (2002) Dairy consumption, obesity, and the insulin resistance syndrome in young adults. *JAMA* **287**, 2081-2089

23. Zemel, M. B., Kim, J. H., Woychik, R. P., Michaud, E. J., Kadwell, S. H., Patel, I. R. and Wilkison, W. O. (1995) Agouti regulation of intracellular calcium: role in the insulin resistance of viable yellow mice. *Proc. Natl. Acad. Sci. USA* **92**, 4733-4737

24. Kim, J. H., Kiefer, L. L., Woychik, R.P., Wilkison, W. O., Truesdale, A., Ittoop, O., Willard, D., Nichols, J., and Zemel, M. B. (1997) Agouti regulation of intracellular calcium. Role of melanocortin receptor. *Am. J. Physiol.* **272**, E379-E384

25. Jones,B. H., Kim, J. H., Zemel, M. B., Woychik, R. P., Michaud, E. J., Wilkison, W. O. and Moustaid, N. (1996) Upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. *Am. J. Physiol.* **270**, E192-E196

26. Xue, B. Z., Moustaid, N., Wilkison, W.O., and Zemel, M. B. (1998) The agouti gene product inhibits lipolysis in human adipocytes via a Ca^{2+}-dependent mechanism. *FASEB J.* **12**, 1391-1396

27. Kim, J. H., Mynatt, R. L., Moore, J. W., Woychik, R. P., Moustaid, N., and Zemel, M. B. (1996) The effects of calcium channel blockade on agouti induced obesity. *FASEB J.* **10**, 1646-1652

28. Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2001) 1α, 25-dihydroxyvitamin D3 modulates human adipocyte metabolism via nongenomic action. *FASEB J.* **15**, 2751-2753

29. Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2002) 1α, 25-dihydroxyvitamin D_3 inhibits uncoupling protein 2 expression in human adipocytes. *FASEB J.*. express article 10. 1096/fj.02-0255fje.

PART 2

LITERATURE REVIEW

I. Obesity Epidemic

Obesity is a chronic disease involving genetic predisposition, environmental influences and behavioral aspects and results in significant morbidity, reduced quality of life, discrimination and early mortality (1). Obesity is objectively assessed by body mass index (BMI), which is defined as weight of an individual in kg divided by height squared in meters (kg/m^2). Based on the facts that with increased degree of overweight and obesity there is an increase in morbidity and mortality risk (2-4), the World Health Organization (WHO) and the International Obesity Task Force (IOTF) recommended the classifications of overweight and obesity as follows: overweight (BMI $\geq$ 25.0); pre-obese (BMI 25.0-29.9); class I obesity (BMI 30.0-34.9), class II obesity (BMI 35.0-39.9), and class III obesity (BMI $\geq$ 40) (5).

Obesity has reached epidemic proportions in the United States and is threatening to become a global epidemic (6). Data from the latest NHANES conducted in 1999 and 2000 show that 64.5% of U.S. adults are now overweight, and 30.5% are obese compared to only 14.5% in 1980 and 22.9% in NHANES III (1988-1994) (7). The prevalence of overweight and obesity has risen dramatically over the past two decades, and the increases are continuing. If this trend persists, the entire U.S. adult population could be overweight or obese within a few generations (8). This alarming increase is also present among the nation's youth: ~15% of U.S. children and adolescents are overweight (9). Overweight that begins early in life persists into adulthood and increases the risk of obesity-related conditions later in life (10). Furthermore, the prevalence has been increasing in varying degrees, not only in the United States, but also in Britain and

elsewhere in Europe, as well as in countries as diverse as Australia, Brazil, China, Mauritius and Western Samoa (5).

Obesity represents a serious threat to health and quality of life because it is strongly associated with an increased incidence of non-insulin-dependent (type II) diabetes mellitus, coronary heart disease, hypertension, stroke, dyslipidemia, and several types of cancer (11). Perhaps one of the most dramatic and disturbing findings in the past decade was that of Pinhas-Hamiel et al. in 1996 (12). After analyzing 1027 patients aged 0-19 y who were diagnosed with diabetes in Cincinnati, they found a tremendous increase (from 4% to 33%) in the incidence of type II diabetes in children and adolescents between 1982 and 1994. In addition to family history and ethnicity (greater risk in African Americans), overweight was identified as a major risk factor for type II diabetes. This study was important because it showed that type II diabetes was not necessarily a slowly progressing disease that affected adults but, in susceptible individuals, could be manifested as early in life as adolescence, and it clearly emphasized overweight as substantially more than a body weight issue in children. Other obesity comorbidities include osteoarthritis and gout, gallbladder disease, and pulmonary diseases, including sleep apnea (11). Obesity-related diseases cause more than 300,000 deaths annually in the US alone (13).

The epidemic of obesity imposes a heavy burden on the healthcare systems of many industrialized countries. Obesity-related morbidity may account for 6.8% of US health care costs (14). The US Surgeon General's Report estimates that the economic cost of obesity in the United States was about $117 billion in 2000, including both direct medical costs and indirect costs such as lost productivity.

Genetic factors appear to play an important role in the regulation of body weight and body fat (15). However, the current epidemic of obesity appears to be caused largely by an environment that promotes excessive food intake and discourages physical activity in genetically susceptible individuals (16). Control of portion size, consumption of a diet low in fat and energy density, and regular physical activity are behaviors that protect against obesity, but it is becoming difficult to adopt and maintain these behaviors in the current environment.

Obesity increases the risk of preventable death by 50% to 150% (17). Weight loss itself is likely to be beneficial. Many studies have shown that during periods of weight loss there is a uniform improvement in the profile of chronic disease risk factors (18, 19). A moderate weight loss (5%~10%) in people who were 50% to 100% overweight produced significant drops in their blood pressure, blood levels of triglycerides, and improved glucose tolerance as well. Moderate weight loss is also associated with significant decreases in total cholesterol and increases in high-density lipoprotein (HDL) cholesterol levels, and subsequently an improvement in the ratio of total cholesterol to HDL cholesterol. In a large 12-yr prospective study of 43,457 overweight middle-aged white women, those who intentionally lost weight and also had obesity-related health problems had a 20% reduction in all-cause mortality (20). This was primarily due to a 40% to 50% decline in obesity-related cancers, such as those of the uterus and cervix, as well as a 30% to 40% decrease in diabetes-associated mortality. In women with no preexisting illness, intentional weight loss of ≥20 lb was associated with about a 25% reduction in all-cause, cardiovascular, and cancer mortality.

Methods for the treatment of obesity include dietary management, physical activity and exercise, and antiobesity drugs, with gastrointestinal surgery being reserved for extreme cases (11). Americans spend about $40 billion per year on weight-loss treatments, mostly in the form of diets and dietary foods (17).

II. Etiology of Obesity

Obesity appears as a complex multi-factorial disease resulting from a prolonged imbalance between energy intake and energy expenditure, especially in genetically susceptible individuals (21). Available evidence suggests that obesity results from multiple interactions between genes and environment. Parental obesity is the most important risk factor for childhood overweight. Single gene defects leading to obesity have been discovered in animals and, in some cases, confirmed in humans as congenital leptin deficiency or congenital leptin receptor deficiency. However, in most cases, genes involved in weight gain do not directly cause obesity, but instead increase the susceptibility to fat gain in subjects exposed to an unfavorable environment such as constant access to energy-dense food and minimal physical demands of daily living (22).

A. Genetic factors

There is strong evidence that genetic factors contribute to the development of obesity in humans as well as laboratory animals. Twin, adoption, and family studies have now established that an individual's risk of obesity is increased when he or she has relatives who are obese. Other studies have shown consistently that ~40 to 70% of the variation in obesity-related phenotypes, such as BMI, sum of skinfold thickness, fat mass, and leptin

levels, is heritable (23). Human obesity is a polygenic trait, being determined by interaction of several "susceptibility" genes, each of which may have relatively small effects, with each other as well as with environmental factors such as nutrients and physical activity (24).

1. Genetic influences on obesity in rodent models

The genes associated with several monogenic defects that produce obesity in experimental rodents have been cloned and characterized. These genes and gene products reveal novel molecular and biochemical mechanisms underlying the etiology of obesity and provide promising targets for therapeutic intervention (25, 26). Several transgenic and knockout rodent models that modify body fat provide insight into important systems that are involved in the regulation of food intake, fat storage and/or energy expenditure (26). The polygenic obese rodent models are excellent models to investigate the genetic mechanisms underlying the development of human obesity involved in gene-gene and/or gene-nutrient interactions (26).

a. Single gene defects

The major monogenic obese rodent models include obese ($Lep^{ob/ob}$) mouse, diabetes ($Lepr^{db/db}$) mouse, Zucker fatty ($Lepr^{fa/fa}$) rat, yellow obese (A^y/a) mouse, fat (Cpe^{fat}/ Cpe^{fat}) mouse and tubby (*tub/tub*) mouse. Of these, the phenotype of the yellow obese (A^y/a) mouse is caused by autosomal dominant mutation while the rest are caused by autosomal recessive mutations.

(1) The obese (*Lep*$^{ob/ob}$) mouse, diabetes (*Lepr*$^{db/db}$) mouse and Zucker fatty (*Lepr*$^{fa/fa}$) rat

The phenotypic expression of the obese syndrome is similar across the three autosomal recessive mutation models. These animals exhibit hyperphagia, morbid obesity and type II diabetes, decreased energy expenditure, insulin resistance/hyperinsulinemia and reproductive deficiency (27). The obese (*Lep*$^{ob/ob}$) mouse and diabetes (*Lepr*$^{db/db}$) mouse are two most widely studied models as the result of Coleman's elegant series of parabiosis experiments (28). *Lep*$^{ob/ob}$ mice whose peripheral circulation was joined surgically with control mice exhibited normalization of body weight. This suggested that *Lep*$^{ob/ob}$ mice were deficient in a circulating factor, now known as leptin or OB protein, which regulated the size of the body fat depot. Alternatively, *Lepr*$^{db/db}$ mice, when linked to their lean controls, did not exhibit weight loss, suggesting that *Lepr*$^{db/db}$ mice were defective in the receptor for the circulating factor leptin.

The mouse *ob* gene, located on mouse chromosome 6, was cloned in 1994 (29). It encodes leptin (OB), which contains 167 amino acids. In the obese (*Lep*$^{ob/ob}$) mouse, a single base substitution at codon 105 was identified. This introduces a stop codon, resulting in a premature-truncated inactive protein. The mouse *db* gene is located on mouse chromosome 4 and encodes the leptin receptor (OB-R) (30-33). This receptor has at least six alternatively spliced forms. OB-R_L, the long form OB-R, has a long cytoplasmic region containing several motifs required for leptin signal transduction, and is expressed at high levels in hypothalamus. The other forms lack some or all of these motifs. They are found at high levels in the choroids plexus, thus are speculated to play a role in transporting leptin from the blood into cerebral spinal fluid (CSF), where it can then move by diffusion to the brain centers that regulate body weight since leptin enters

the brain by a specific and saturable transport mechanism (34). In *Lepr*$^{db/db}$ mice, a G→T point mutation was identified in the genomic OB-R sequence, which generates a donor splice site that converts a 106 nucleotide region with a premature stop codon into a novel exon transcript (30). This abnormal splicing resulted in a short form OB-R without the long intracellular domain. Thus, the long intracellular domain of OB-R is crucial for initiating leptin signal transduction. A similar mutation is thought to be present in Zucker fatty (*Lepr*$^{fa/fa}$) rat (35, 36).

Leptin is produced primarily in white adipose tissue. Both peripheral and central administration of recombinant leptin in *Lep*$^{ob/ob}$ mice lowered their body weight, percent body fat, food intake, concentrations of glucose and insulin; increased metabolic rate, body temperature, activity levels; and repaired reproductive function but had no effect on *Lepr*$^{db/db}$ mice (37-40). Thus, leptin serves an endocrine function to regulate body fat stores, and it can act directly on neuronal networks that control feeding and energy balance. The hypothalamus is the primary target tissue which leptin act upon to exert its energy-regulatory effects (41, 42). Hypothalamic neuropeptide Y (NPY) stimulates food intake, decreases thermogenesis and increases plasma insulin and corticosterone levels. Leptin has been shown to exert its weight reducing effects via inhibition of NPY synthesis and release (43). Thus, leptin responsive neurons in hypothalamus express neuropeptides that are modulated by leptin and interact with each other in controlling feeding behavior and energy balance.

The mouse *ob* gene is highly homologous with human, monkey and rat *ob* genes. In contrary to the leptin deficiency found in the obese (*Lep*$^{ob/ob}$) mouse, leptin gene expression increases with body fat in a range of obese animal models, including the

diabetes (*Lepr*$^{db/db}$) mouse, the Zucker fatty (*Lepr*$^{fa/fa}$) rat, and rats fed a high-fat diet (44). Several studies have examined leptin in a variety of obese and normal humans as well. A generally positive correlation between fat mass and leptin levels is observed in individuals with extreme obesity, suggesting that leptin deficiency is not a common cause of obesity in human populations (45, 46). This has led to the suggestion that human obesity may be caused by resistance to leptin action. As mentioned earlier, the transport of leptin across the blood/brain barrier may be saturable, as leptin levels in human CSF are normally <10% of serum levels (34, 47). Another possibility is that significant numbers of humans will have a polymorphism in the OB-R gene, which results in the production of receptors with different signaling potency (48). More likely, leptin resistance may be a result of flaws within the signal-transducing pathway of the leptin receptor, more analogous to the mechanism of insulin resistance (33).

(2) The yellow obese (A^y/a) mouse

Dominant mutations in the mouse *agouti* gene confer a pleitropic syndrome characterized by maturity-onset obesity, mild hyperphagia, decreased thermogenesis, hyperinsulinemia, peripheral insulin resistance, impaired glucose tolerance, increased linear growth and yellow fur (49, 50). The *agouti* gene, located on mouse chromosome 2, was the first obesity gene cloned and characterized (51, 52). It encodes a 131-amino-acid protein with a consensus signal peptide. Agouti protein is normally transiently expressed in the hair follicle during the mid-portion of the hair growth cycle. It acts to inhibit the synthesis of eumelanin, a black pigment, by competitively antagonizing α-melanocyte-stimulating hormone (α-MSH) binding to its melanocortin-1 receptor (MC1-R), resulting

in a down-regulation of cAMP production and a temporary synthesis of phaeomelanin, a yellow pigment. This produces the characteristic pigmentation pattern of wild-type mice, a predominantly black hair shaft with a subapical yellow segment (53). In the yellow mouse, dominant mutations in promoter regions result in ubiquitous expression of normal agouti protein in a wide range of tissues, including white adipose tissue and hypothalamus (51), throughout the life of the mouse. Transgenic mice ubiquitously expressing agouti driven by the human β-actin promoter also develop yellow coat color and become obese (54, 55), confirming that ectopic expression of normal agouti protein in the obese yellow phenotype. Interestingly, the human homolog of the agouti gene, named agouti signaling protein (ASIP) that is 85% homologous to mouse agouti, is expressed primarily in adipose tissue and pancreas (56), suggesting that it may play a role in human obesity.

Although the genetic defect in the agouti yellow mouse syndrome clearly involves ectopic expression of the agouti gene, the actual mechanism of the yellow obese syndrome is not clear. Both central and peripheral effects of agouti have been explored extensively (57). Agouti is a potent antagonist of the melanocortin-4 receptor (MC4-R), a related MSH-binding receptor MC1-R (58, 59). MC4-R is widely expressed throughout the brain, with a high degree of expression in hypothalamus centers involved in appetite and body weight regulation (60). Intracerebroventricular administration of a potent MC4-R agonist inhibited food intake in four murine models of obesity and hyperphagia while co-administration of the specific MC4-R antagonist completely blocked this inhibition (61). Further, MC4-R knockout mice exhibit adult-onset obesity associated with hyperphagia, hyperinsulinemia and insulin resistance, recapitulating many of the

metabolic features of the yellow obese mouse (62). Thus, the chronic antagonism of MC4-R may be responsible for the development of the yellow mouse obesity. An accumulating body of evidence (63-70) indicates that peripheral actions of agouti are likely to contribute to agouti-induced obesity as well, and strongly suggest a role of agouti signaling in adipocytes and pancreatic β-cells through its ability to increase intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels, and consequently stimulate triglyceride storage and inhibit lipolysis, as discussed in detail later.

The yellow mouse exhibits maturity-onset obesity, which is primarily due to increased metabolic efficiency, with a preferential partitioning of food energy into fat storage (57). Yellow mice exhibit increases in the expression and activity of lipogenic enzymes in both liver and adipose tissue (66, 71). Further, lipolysis is reduced in adipose tissue of yellow mice compared to wild-type mice and the lipolytic response to epinephrine is suppressed as well (72). Thus, the coordinated augmentation of lipogenesis and inhibition of lipolysis observed in these mice may contribute to the increase in metabolic efficiency. Given the role of insulin in promoting nutrient partitioning into adipose tissue, hyperinsulinemia may contribute to the enhanced metabolic efficiency of yellow mice (57). There is positive correlation between weight gain and insulinemia and between insulin levels and lipogenic enzyme activity in obese yellow mice (71). However, hyperinsulinemia is not sufficient to produce the obesity in the absence of agouti expression. Transgenic mice expressing agouti at high levels in adipose tissue under the control of the adipose tissue-specific fatty acid-binding protein aP2 promoter (aP2-agouti transgenic mice) become obese if they are also hyperinsulinemia while hyperinsulinemia was without effect in non-transgenic littermate

controls (73). In fact, agouti and insulin appear to exhibit a synergistic interaction, which results in the development of obesity (74, 75). Recent data indicate that these effects are mediated by modulation of $[Ca^{2+}]_i$ (63-70).

(3) The fat (*Cpe*^{fat}/*Cpe*^{fat}) mouse

The fat mouse exhibits progressive adult-onset obesity and is characterized by massive hyperinsulinemia (76). The apparent hyperinsulinemia was in fact a hyperproinsulinemia (77). The *fat* gene, located on mouse chromosome 8, has been shown to encode carboxypeptidase E (CPE), which is required for cleavage of two arginine residues from the B chain of insulin during its processing from proinsulin. In addition to proinsulin, a number of prohormones and proneuropeptides, including pro-opiomelanocortin (POMC), also require cleavage of paired dibasic residues from their C-terminal ends to yield the biologically active peptides. POMC is a precursor for peptides such as adrenocorticotropic hormone (ACTH) and MSH, and MSH is known to act on the melanocortin receptor family involved in the control of feeding (78). In the fat mouse, a single base mutation that results in a serine to proline substitution at position 202 severely reduces the amount and activity of CPE in both the pancreatic islet and pituitary (77). As CPE is expressed in central nervous system (CNS), the obesity phenotype of fat mouse is likely to result from defects in the production and activity of neuropeptides such as POMC in hypothalamus (77-79).

(4) The tubby (*tub/tub*) mouse

Homozygous mice with a mutation at the *tub* locus develop late-onset obesity and insulin resistance (76, 80). The *tubby* gene, located on mouse chromosome 7, was cloned in 1996 (80, 81). In *tub/tub* mouse, a single base mutation of G→T within a splice donor site in the 3' coding region abolishes the donor splice site and results in a larger transcript containing the unspliced intron. The consequence of this mutation is the substitution of the 44-carboxy-terminal amino acids with a 20-amino-acid sequence not found in the wild-type protein. It was shown that tubby proteins localize to the plasma membrane by binding phosphatidylinositol 4, 5-bisphosphate through its carboxyl terminal "tubby domain", and function as membrane-bound transcription factors that translocate to the nucleus in response to receptor-mediated activation of GTP-binding protein (G protein) (82). The normal tubby proteins appear to be abundantly expressed in the hypothalamus, suggesting that obesity in the tubby mice may stem from malfunction of signaling pathways in the satiety center (81).

b. Transgenic and knockout rodent models of obesity

Table 1 lists several transgenic and knockout rodent models that have been shown to modify body fat. Each of these transgenic animals provides some insights into important systems that are physiologically perceived to be involved in the regulation of food intake, fat stores and energy expenditure.

Available evidence indicates that the hypothalamic-pituitary-adrenal axis (HPA) plays a key role in the control of energy balance in laboratory rodents (83). Transgenic

Table 1. Transgenic and Knockout Rodent Models of Obesity

Gene	Tg or KO*	Major tissues	Phenotypes	References
CRH*	Tg	CNS*, testis, heart, adrenal gland	Cushing's syndrome	84
GCR*	KO	CNS, liver	Impaired GCR function; obesity	85
GLUT4*	Tg	AT*	Obesity due to adipocyte hyperplasia; enhanced glucose disposal	91
Agouti	Tg	Ubiquitous	Reproduced phenotype of A^y/a obese yellow mice	55
	Tg	AT	Become obese if hyperinsulinemia	73
MC4-R*	KO	CNS	Recapitulated features of A^y/a obese yellow mice	62
POMC*	KO	CNS	Obesity, hyperphagia; adrenal Insufficiency; altered pigmentation	92
UCP1*	UCP1-DTA* expression	BAT*	Obesity with hyperphagia; ablated BAT; impaired BAT thermogenesis	96
	KO	BAT	Cold-sensitive but not obese	101
UCP2*	KO	AT, SM*, immune system	Not obese, not cold-sensitive; macrophages generate more ROS; resistant to infection	115
	Tg	normal rat pancreatic islets	Impaired glucose-stimulated insulin secretion	118
	Tg	ZDF rat* pancreatic islets	Enhanced insulin secretion	119
UCP3*	Tg	SM	Hyperphagic but lean; increased resting metabolic rate; improved insulin sensitivity	107
	KO	AT, SM	Not obese; more coupled; increased production of ROS*	108
β_3-AR*	KO	AT	Modest obesity	132
β-ARs*	KO	AT	Massive obesity during overfeeding due to failed DIT*	133

* Tg: transgenic; KO: knockout; CRH: corticotropin-releasing hormone; CNS: central nervous system; GCR: glucocorticoid receptor; GLUT4: GLUT 4 glucose transporter; AT: adipose tissue; MC4-R: melanocortin-4 receptor; POMC: pro-opiomelanocortin; UCP1-3: uncoupling protein 1-3; DTA: diphtheria toxin A-chain; BAT: brown adipose tissue; SM: skeletal muscle; ZDF rat: Zucker Diabetic Fatty rat; ROS: reactive oxygen species; β_3-AR: β_3-adrenergic receptor; β-ARs: β_1, β_2, β_3-adrenergic receptors; DIT: diet-induced thermogenesis

mice overexpressing the corticotropin-releasing hormone (CRH) gene under control of the metallothionein promoter exhibit endocrine abnormalities involving the HPA axis, such as elevated plasma levels of ACTH and glucocorticoids (84). These animals display features of Cushing's syndrome with truncal obesity, decreased linear growth, marked bilateral hair loss, muscle wasting and reduced fertility.

The type II glucocorticoid receptor (GCR) is involved in glucocorticoid negative-feedback effects on the HPA axis activity. Barden et al (85) incorporated a type II GCR antisense RNA construct into mice. The transgenic mice had greatly increased fat deposition and, at ~6 months of age, were twice the weight of normal mice. Northern blot analysis of endogenous type II GCR mRNA indicates a maximal 50-70% decrease in hypothalamus and cerebral cortex, and a smaller 30-55% decrease is seen in liver. The transgenic mice also have a reduced GCR binding activity for glucocorticoids with raised serum ACTH and glucocorticoids concentrations due to a failure of glucocorticoids to inhibit HPA axis activity. Glucocorticoids can decrease the number of GCRs in both hypothalamus and pituitary glands, and the high glucocorticoid levels in transgenic mice, secondary to decreased type II GCR levels, could be an additional factor in downregulating the type II GCR even further compared with antisense RNA (85). This is consistent with previous studies emphasizing the dependence of all obesities on the presence of adrenal glucocorticoids in a wide range of animal models, including the genetically obese $Lepr^{fa/fa}$ rats, $Lep^{ob/ob}$, $Lepr^{db/db}$, and A^{y}/a mice. Bilateral adrenalectomy prevents, attenuates or reverses the development of obesity in these animals (86-89), suggesting that glucocorticoids are required for the phenotypic expression of genetic obesity.

The GLUT 4 glucose transporter plays a pivotal role in insulin-stimulated glucose transport in adipose tissue and muscle (90). Overexpression of GLUT 4 in white adipose tissue (6-9-fold) and brown adipose tissue (3-5-fold) using the aP2 promoter/enhancer increased total body lipid 2-3-fold (91). This was associated with a massive increase in the basal transport of glucose into adipocytes and, interestingly, a twofold increase in fat cell number, but no increase in fat cell size. This provided the first model of obesity associated with hyperplastic growth of adipose tissue in the absence of cell hypertrophy. Thus, it is a valuable model for understanding the mechanisms responsible for adipocyte replication and/or differentiation *in vivo.*

Mice in which the POMC gene has been inactivated exhibit obesity and hyperphagia. The mice also exhibit adrenal insufficiency owing to a lack of ACTH, and altered pigmentation owing to a lack of MSH (92). The mice exhibit a phenotype that is essentially identical to that observed in humans with inactivating mutations in the gene encoding POMC (93). This model will undoubtedly prove useful in determining the roles of the individual cleavage products of POMC on energy homeostasis.

Uncoupling protein-1 (UCP1) is a mitochondria proton transporter expressed exclusively in mammalian brown adipose tissue (BAT). UCP1 dissipates the proton electrochemical gradient across the mitochondria membrane, thereby uncoupling substrate oxidation from conversion of ADP to ATP, leading to generation of heat and thus increased energy expenditure (94, 95). As BAT and UCP1 are abundant in rodents, BAT thermogenesis may be important in the regulation of energy expenditure in rodents. Dysfunction of BAT could lead to decreased energy expenditure and could play a critical role in the pathogenesis of obesity in rodents. Indeed, Lowell et. al created transgenic

mice in which the UCP1 promoter drives BAT specific expression of diphtheria toxin A-chain (UCP-DTA), thus causing primary deficiency of BAT (96). In these transgenic mice, both UCP1 content and BAT was markedly decreased. Correspondingly, BAT thermogenic function was abolished in response to both cold and CL316,243, an extremely selective β3-adrenergic receptor agonist. Moreover, these mice became obese and developed hyperphagia, illustrating the possible link between BAT thermogenic function, food intake and obesity in rodents. Interestingly, hyperphagia and obesity are absent when UCP-DTA mice are reared at thermoneutrality, which indicates that even in this model, there is no obesity without hyperphagia (97). Bray et al. demonstrated a highly significant inverse relationship between spontaneous electrical activity of sympathetic nerves innervating BAT and food intake in spontaneously feeding rats, suggesting the reciprocal relation between BAT thermogenesis and food intake (98, 99). Accordingly, Himms-Hagen proposed a thermoregulatory feeding hypothesis (100). That is, the initiation of feeding occurs during an episode of increased sympathetic nervous system activity that stimulates BAT thermogenesis and increases body temperature. Once body temperature increases to a certain level, the feeding terminates. Thus, BAT thermogenesis is important in control of meal size and satiety. When this function is lost, the animal loses its ability to control meal size, and hyperphagia and obesity occur subsequently.

Contrary to this thermoregulatory feeding hypothesis is another study conducted by Enerbäck et al., demonstrating that mice lacking mitochondria UCP1 are cold-sensitive but not hyperphagia and obese (101). UCP1 knockout (KO) mice were generated by target inactivation of UCP1 gene. The BAT of UCP1 KO mice has enlarged lipid

vacuoles but in all other respects it appears normal. The oxygen consumption after treatment with CL316,243 was significantly blunted in UCP1 KO mice compared to wild-type mice, like that found for UCP-DTA mice. UCP1 KO mice are more cold-sensitive than UCP-DTA mice, indicating that their thermoregulation is defective. As it is assumed that a role for BAT in energy balance is determined by UCP1-derived thermogenesis, UCP1 KO mice should be as, or more obese than, UCP-DTA mice. Unexpectedly, the knockout of UCP1 caused neither hyperphagia nor obesity in mice fed on either standard or a high-fat diet. To explain this unexpectancy, the investigators proposed that an alternative mechanism for maintaining body mass must exist that cannot protect against the cold. They found that UCP2, a recently discovered homologue of UCP1, was up-regulated fivefold in the BAT of UCP1 KO mice. Thus, UCP2 may compensate for the loss of UCP1 to maintain normal body mass without protecting against cold.

Taken these studies together, UCP 1 is important for thermogenesis while BAT plays a critical role in the regulation of energy balance in mice, and that BAT deficiency can cause obesity.

UCP2 (102, 103) and UCP3 (104, 105) are more recently discovered members of the mitochondria inner membrane transporter family with high homology (59% and 57%, respectively) to the BAT-specific UCP1. UCP3 is expressed primarily in skeletal muscle and BAT while UCP2 is widely expressed but at varying levels in a number of tissues and cell types including skeletal muscle, BAT, WAT, brain, lung, heart, kidney, placenta, spleen, leukocytes, macrophage etc. Both *in vitro* and *in vivo* studies support the uncoupling activity of these proteins indicated by high proton leak and low mitochondria

membrane potential (102, 103, 106-108). However, their physiological roles are unknown. It is well established that BAT is an important tissue for thermogenesis in rodents. However, in large mammals in which BAT is less common, skeletal muscle may be more important for thermogenesis (109). Thus, based upon their high homology with UCP-1 and their tissue distribution in skeletal muscle and adipose tissue, UCP2 and UCP3 have been suggested to play important roles in regulating energy expenditure, body weight and thermoregulation (102, 105). UCP2 and UCP3 are also implicated in the regulation of lipids as fuel substrate in skeletal muscle on the basis that their mRNA expressions are up-regulated during starvation (when fat stores are being rapidly mobilized) and down-regulated during the early phase of re-feeding (when fat stores are being rapidly replenished) (110, 111). These starvation/re-feeding studies suggest that UCP2 and UCP3 may function as regulators of lipids as fuel substrate rather than as mediators of regulatory thermogenesis. It has also been proposed that UCP2 and UCP3 could prevent the formation of oxygen-free radicals in skeletal muscle. Several studies show that reactive oxygen species (ROS) production is a function of the mitochondria membrane potential. It has been hypothesized that ROS are produced when the mitochondria membrane potential is above a specific threshold. Mild uncoupling of the mitochondria could be a mechanism to prevent the formation of ROS (112-114). This mechanism could be important in skeletal muscle, given that it accounts for an important fraction of the total oxygen consumption at rest and that this fraction is even higher when skeletal muscle switches to maximal activity. Thus, one of the functions of UCP 2 and UCP3 could be to prevent excessive oxidative stress in skeletal muscle (108). In summary, the information available about the physiological role of UCP2 and UCP3 is

still controversial and incomplete. To further illustrate the physiological function of these two uncoupling proteins, both overexpression and knockout rodent models have been generated.

Clapham et al. created transgenic mice that overexpress human UCP3 driven by α-skeletal actin promoter in skeletal muscle (107). These mice are hyperphagic but weigh less than their wild-type littermates. Magnetic resonance imaging shows a striking reduction in adipose tissue mass. This phenotype is associated with increased resting metabolic rate as measured by 25% to 40% higher resting oxygen consumption than the wild-type littermates. The mice also exhibit lower fasting plasma glucose and insulin levels and an increased glucose clearance rate, indicating improved insulin sensitivity. This provides evidence that skeletal muscle UCP3 has the potential to influence metabolic rate and glucose homeostasis in the whole animal, suggesting that enhancement of UCP3 expression or stimulation of its activity is a promising approach for the treatment of obesity.

Vidal-Puig et al generate UCP3 gene knockout (KO) mice using a gene targeting approach (108). These mice do not develop obesity. They found that skeletal muscle mitochondria lacking UCP3 are more coupled, indicating that UCP3 has uncoupling activity. They also found that there is increased production of ROS in skeletal muscle mitochondria lacking UCP3. They then demonstrate that UCP3, expressed at endogenous levels, has uncoupling activity and its absence may lead to increased production of ROS, consistent with the hypothesis that UCP3 may function to prevent excessive oxidative stress in skeletal muscle. Their data also show that UCP3 does not seem to be required for normal body weight regulation. This observation may not be completely surprising,

since the genetic disruption of UCP1 did not result in obesity (101). However, unlike UCP1 KO mice, mice lacking UCP3 are not cold sensitive, and they do not have up-regulation of either UCP2 or UCP1 mRNAs. These suggest that UCP3 may not play an important role in regulating body weight or adaptive thermogenesis in response to cold exposure. However, it can not be excluded that the lack of UCP3 activity could be compensated through post-translational activation of UCP2 and/or UCP1 activity or, alternatively, through activation of other unknown futile cycles. Thus double and triple UCP KO mice would be useful tools to address these questions.

Similarly, Mice lacking UCP2 following targeted gene disruption are not obese and have a normal response to cold exposure or high-fat diet (115). Moreover, they are completely resistant to infection, in contrast with the lethality observed in wild-type littermates. Macrophages from UCP2 KO mice generated more ROS than wild-type mice, indicating a role for UCP2 in the limitation of ROS and macrophage mediated immunity. Since there are high levels of UCP2 mRNA expression in organs of the immune system such as spleen, lung and macrophages (102, 103), a role for UCP2 in immunity or inflammatory responsiveness makes sense. The authors also found increase of UCP1 mRNA expression in WAT. Increase of UCP2 mRNA was previously noted in BAT of mice deficient for UCP1 (101). Thus, this study suggests that UCP1, but not UCP2, may play a critical role in both cold- and diet-induced thermogenesis and regulation of body weight. UCP2 may be important to limit ROS production by its mild uncoupling activity. Between UCP2, immunity and ROS, UCP2 KO mice now provide a new tool to further elucidate molecular mechanisms of resistance to infection.

UCP2-overexpressing rats were used to understand the relationship between UCP2 and β-cell insulin secretion. As UCP2 rat homolog is present in pancreatic islets, among other tissues (116, 117), two different groups created rats overexpressing UCP2 in pancreatic islets. Chan et al. overexpressed human UCP2 in normal rat islets by infection with an adenovirus (AdEGI-UCP-2) (118). The mice exhibited severe blunting of glucose stimulated insulin secretion (GSIS), illustrating a direct, causal relationship between overexpression of UCP2 and inhibition of GSIS in normal islets. The mechanism by which UCP2 affects insulin secretion is predicted to be via decreasing cellular ATP content. However, in another study conducted by Wang et al., UCP2 overexpression in islets of Zucker Diabetic Fatty (ZDF) rat reduced β-cell ATP but increased the ATP/ADP ratio and enhanced insulin secretion in ZDF rat islets (119). Thus, results to date in normal and ZDF rats appear contradictary, but a possible mechanism for reduction of GSIS during starvation and type II diabetes might include upregulation of UCP2 expression (120).

The sympathetic nervous system (SNS) influences many physiological functions including body temperature homeostasis and lipid metabolism by releasing the neurotransmitter norepinephrine that acts upon β-adrenergic receptors (β-ARs)(121). All three known subtypes of β-ARs, namely β_1-, β_2- and β_3-AR, are expressed in both WAT and BAT. They all couple via G_α-protein to adenylate cyclase (AC), leading to an increase in cAMP. The resulting activation of protein kinase A (PKA) mediates the major actions of these receptors that include stimulation of lipolysis in white adipocytes and thermogenesis in brown adipocytes (122).

β_3-ARs are expressed predominantly in WAT and BAT in mice (123, 124). Moreover, a number of *in vivo* studies have provided clues regarding the possible physiological significance of β_3-ARs. In genetically obese $Lepr^{fa/fa}$ rats (124) and $Lep^{ob/ob}$ mice (125), β_3-ARs mRNA levels are significantly down-regulated, raising the possibility that decreased β_3-ARs function might contribute to the development of obesity in these animals. Selective activation of β_3-ARs leads to marked increases in triglyceride breakdown (lipolysis) and energy expenditure as measured by effects on oxygen consumption, and long-term treatment of obese rodents with β_3-selective agonists reduces fat stores and improves obesity-induced insulin resistance (126-128). Of note, it has been reported that a missense mutation of the human β_3-ARs tends to be associated with obesity, decreased energy expenditure, reduced insulin sensitivity and earlier onset of type II diabetes (129-131). These observations suggest that β_3-ARs might also play an important role in humans as well. Thus, β_3-ARs represent one of a number of potential anti-obesity drug targets (126-128). Susulic et al. generated β_3-AR-knockout mice (β_3-KO) by direct injection of a DNA targeting construct into mouse zygotes (132). The β_3-KO mice had normal body weight compared to wild-type mice, although they do exhibit modestly increased fat stores (females more than males). Interestingly, fed insulin and glucose levels and food intake are unchanged in the β_3-KO mice, indicating that compensatory mechanisms might operate in these animals to maintain normal energy homeostasis. In fact, the expression of β_1-AR mRNA is upregulated in WAT and BAT in these animals. Finally, acute treatment of normal mice with CL316,243, a selective β_3-agonist, increased serum levels of free fatty acids (FFAs) (3.2-fold) and insulin (140-

fold), increased energy expenditure (2-fold) and reduced food intake (by 45%). However, these effects were completely absent in β_3-KO mice, proving that the actions of CL are mediated exclusively by β_3-AR. Thus, this β_3-KO mouse model should be useful as a means to a better understanding of the physiology and pharmacology of β_3-ARs.

Recently, Bachman et al. created mice lacking all three β-ARs (β-less mice) and provided direct evidence that the SNS and the β-AR signaling pathways are necessary for diet-induced thermogenesis (DIT) (133). Previous overfeeding experiments indicated that humans vary in their capacity to resist weight gain because of varying abilities to convert food directly into heat, a process called DIT (134). Further, Landsberg et al. demonstrated that SNS activity in a variety of tissues is boosted during overfeeding and decreased during starvation (a state of energy conservation), suggesting the SNS as the efferent system linking diet and thermogenesis (135). Bachman et al. showed that the β-less mice on a Chow diet had a reduced metabolic rate and were slightly obese. On a high-fat diet (58% of kcal from fat), the β-less mice could not increase thermogenesis and became massively obese. In contrast, the wild-type mice are able to resist obesity during overfeeding by activating DIT. Thus, β-less mice have a failure of DIT. In addition, the β-less mice are also intolerant to cold exposure, suggesting that the SNS-β-AR pathway control heat production in response to both diet and cold. BAT, because of its UCP1-mediated uncoupling of respiration and its intense sympathetic innervation, is thought to be the source of DIT. Indeed, BAT in β-less mice was unresponsive to both physiological (cold exposure) and pharmacological (β-agonist) stimulation. Compared to wild-type mice, BAT in β-less mice expressed lower levels of UCP1 at room temperature and failed

to induce UCP1 expression after cold exposure. A maximally effective dose of isopreterenol, a nonselective β-agonist, stimulated oxygen consumption in wild-type mice more than twofold but had no effect in β-less mice. Thus, β-ARs are necessary for normal BAT function. Both supporting and opposing evidence exist regarding the role of BAT in DIT. Transgenic mice in which UCP1 promoter drives BAT specific expression of diphtheria toxin-A (UCP1-DTA), which have markedly ablated BAT, are obese and sensitive to DIT (96). However, UCP1 gene-knockout (UCP1 KO) mice, although showing greatly impaired cold-induced thermogenesis, are neither obese nor sensitive to diet-induced obesity (101). Thus, UCP1 is required for cold- but not diet-induced thermogenesis whereas β-ARs are required for both. In summary, the β-less mouse model indicates that β-ARs are necessary for DIT and that the SNS-β-AR pathway plays a critical role in the body's defense against diet-induced obesity.

c. Polygenic models of obesity

The polygenic forms of obesity, which are normally of later onset and sometimes are less severe, are often influenced by gene-gene and/or gene-diet interactions (44). The two most studied polygenic mouse models are the AKR mouse model and the BSB mouse model (26). Inbred mouse strains, produced by 20 or more generations of brother × sister matings, provide the basic resource for analysis of multigenic traits (136). West et al. developed and characterized a polygenic model of differential susceptibility to dietary obesity in the inbred mouse strains (137). The AKR/J strain of inbred mouse becomes significantly obese when fed a high-fat diet while the SWR/J strain remains relatively

lean. Intercrosses between these two strains segregate the phenotype of sensitivity to dietary obesity, thus provides a useful tool to pursue the metabolic and genetic basis of this trait under the influence by gene-diet interaction. The BSB mouse model is derived from C57BL/6J mice crossed with *Mus spretus* and the F1 mice subsequently backcrossed onto the C57BL/6J strain (138). A significant percentage of the mice in the backcross generation become obese spontaneously on a low-fat diet with a body fat content that shows individual variation from 1% to 50% while neither of the parental strains demonstrate spontaneous obesity, suggesting that obesity may result from the interactions of two genes.

Quantitative trait loci (QTL) mapping is now a general technique to map all of the genes underlying any complex trait such as obesity by using polymorphic genetic markers covering the whole mouse genome (136). The QTL approach involves the crossing of two strains that differ in the trait of interest to produce F2 or back-cross progeny, individually phenotyping and genotyping each progeny for markers that are spread throughout the genome. Then statistical association of markers and phenotypes are performed to identify loci underlying the trait of interest (136). Warden et al. have applied QTL mapping to spontaneous obesity in the BSB mouse model, with identification of loci promoting obesity on chromosomes 6, 7, 12 and 15 (139, 140). West et al. have also used QTL mapping to identify loci underlying diet-induced obesity in an F2 cross between mouse strains SWR/J and AKR/J, with identification of loci linked to adiposity on chromosomes 4, 9 and 15 (141, 142). So far, over 70 loci for polygenic obesity have been identified from more than 15 different crosses (143).

Since human obesity is polygenic, these polygenic animal models may be used to investigate the genetic mechanisms underlying the development of human obesity involved in gene-gene and/or gene-nutrient interactions.

2. Genetic influences on obesity in humans

There is strong evidence for a genetic component to human obesity. Studies have shown that the prevalence of obesity is 2 to 8 times higher in families of obese individuals than in the population at large, and that the familial risk increases with the severity of obesity. Heritability estimates tend to be highest when derived from twin studies (50% to 80%) while they are the lowest when derived from adoption studies (10% to 30%) (144). Several studies have reported the presence of major gene effects for BMI, body fat and abdominal visceral fat (24).

Monogenic obesity has been described in a few families due to mutations in leptin (145, 146), leptin receptor (147), CPE (148), POMC (93), MC4-R (149-151), prohormone convertase 1 (PC1) (152) or PPARγ (153). For instance, two children with homozygous mutations for loss of leptin function exhibit phenotypes of morbid obesity and hyperphasia (145, 146). Another two children with loss of function mutations in POMC gene exhibit impaired melanocortin signaling and display obesity and altered pigmentation phenotypes (93). Finally, several groups have reported MC4-R mutations (149-151). The metabolic phenotypes caused by MC4-R mutations in humans are similar to those caused by impaired melanocortin signaling in mice. Although single gene mutations as causes of obesity in humans is rare, these mutations confirm that these gene products play a role in human obesity and may allow further elucidation of their signal transduction pathways. Several groups have searched for linkage between obesity-related

phenotypes and the chromosomal region encompassing leptin protein; a meta-analysis suggests that there may be linkage with BMI (154).

The role of genetic factors in common human obesities is complex, being determined by interaction of several susceptibility genes (polygenic), each contributing only small fraction to the overall susceptibility to obesity, with each other and with environmental factors such as diet and physical activity (24). This makes the search of human obesity genes a very challenging task. Two strategies, the QTL mapping approach and the candidate gene approach, are now used to identify human obesity genes (23).

The role of a genetic predisposition to obesity affects both energy intake and energy expenditure. An update of the genetics of human obesity revealed that putative loci affecting obesity-related phenotypes are found on all but chromosome Y of the human chromosomes, and the numbers of genes and markers that have been linked with human obesity are increasing rapidly and now approach 200 (155). Some genes are involved in food intake control (NPY, leptin, POMC, cholecystokinin, melanin-concentrating hormone etc.) or the regulation of energy expenditure and thermogenesis (β-2 and β-3 adrenergic receptors, UCP 1-3, leptin etc.), while the expression of some others influence different signaling pathways, adipogenesis etc. (PPARγ, aP2, PKA, CCAAT-enhancer binding protein etc.), affecting the energy balance (155).

The possible physiological mechanisms through which a genetic susceptibility may be operating include low resting metabolic rate, low rates of oxidation, low fat-free mass and altered food intake, as well as other factors related to macronutrient utilization, energy expenditure or the hormonal profile, including insulin sensitivity (156). For example, UCP2/UCP3 polymorphisms may produce variability in metabolic rates,

thereby producing susceptibility or resistance to obesity (157, 158). Studies of 640 French Canadians and 790 Pima Indians showed that certain DNA sequences that flank the UCP2 gene are found primarily in people with low metabolic rates .

With the publication of the Human Genome, it is likely that our understanding of the genetic basis and the identification of novel genes involved in obesity will be greatly improved. New pathways in the etiology of obesity will be uncovered, and in the more distant future individual identification of genetic variants will be possible, making individually targeted therapies available (159).

B. Environmental factors

On the simplest level, obesity can arise only when energy intake exceeds energy expenditure. The primary environmental factors contributing toward constant positive energy balance are high calorie intake and low levels of physical activity (160). Our current environment is characterized by nearly unlimited supply of convenient, relatively inexpensive, highly palatable, energy-dense foods, coupled with a lifestyle requiring only low levels of physical activity for living. Such an environment promotes high-energy intake and low energy expenditure (16). Egger & Swinburn described our present westernized environment as an 'obesigenic environment' (161).

1. Diet

a. High-fat diets

In Western societies, high consumption of dietary fat has been linked to a number of adverse health outcomes, including cardiovascular disease, some forms of cancer and

obesity (162). Considerable evidence from animal studies, carefully controlled laboratory studies, cross-sectional studies, clinical trials and studies in individuals at high risk to develop obesity clearly demonstrated that consumption of a high fat (HF) diet (≥35% of energy from fat) increases the likelihood of obesity and that the risk of obesity is low in individuals consuming low fat (LF) diets (<20% of energy from fat) (163).

Obesity is rare in experimental animals maintained on a LF diet, even when they are housed in small cages that limit physical activity. In contrast, providing sedentary animals with *ad libitum* HF diets reliably produces increases in energy intake, increases in efficiency of body fat gain, and obesity (164, 165). Dietary obesity can be reversed by switching rodents from a HF to a LF diet, but the extent to which this occurs depends on the severity and duration of the dietary obesity (166).

In numerous human studies, total energy intake was higher when subjects consumed diets relatively high in fat than when they ate lower fat diets (167-169). Moreover, body fat storage occurs at a greater rate when excess energy comes from fat than when it comes from carbohydrate or protein (170). On the other hand, populations consuming very-low-fat diets usually do not show high rates of obesity prevalence. A review of the results from 28 clinical trials that studied the effects of a reduction in the amount of energy from fat in the diet showed that a reduction of 10% in the proportion of energy from fat was associated with a reduction in weight of 16 g/d (171). This is consistent with another behavioral study indicating that dietary restraint provides protection against the obesity-promoting effect of the high-fat, high-energy density diet (172). Additional support for the role of LF diets in regulating body weight comes from studies of formerly obese individuals, a group considered to be at high risk for weight regain. After weight

loss induced by an energy-restricted diet, weight regain at 2 yr was less in subjects assigned to an *ad libitum* LF diet (5.4kg) than those consuming a fixed energy intake diet (11.3kg) (173). Finally, subjects in the National Weight Control Registry, a database of over 2000 individuals successfully maintaining a weight loss of at least 30 pounds for at least 1 yr, report eating a diet low in fat and participating in very high levels of physical activity (174).

Our current food supply is high in fat and HF diets promote obesity by increasing energy intake, thus, increasing the probability of positive energy balance and weight gain. However, the apparent effect of fat per se on energy intake in most studies is difficult to separate from an effect of energy density. Since HF foods have a higher energy density than LF foods, it is possible that the energy density of the diet rather than the dietary fat was responsible for the increased energy intake (16). This hypothesis is supported by several studies indicating that when the fat content but not the energy density of diets varied, fat content did not affect energy intake (175, 176). Conversely, when energy density was manipulated independent of fat content, energy density directly influenced energy intake (177-179). Although there remains controversy over the effects of dietary fat content on energy intake, considering the fact that not all energy-dense foods are high in fat, few HF foods are low in energy density, reductions in dietary fat may still be the most effective means of reducing the dietary energy density, and subsequently the likelihood of excessive energy consumption (16).

b. Portion size

Accumulating evidence suggest that larger food portion sizes may contribute to the increasing prevalence of overweight and obesity (16, 180, 181). Marketplace food portions have increased in size and now exceed federal standards. Portion sizes began to grow in the 1970s, rose sharply in the 1980s, and have continued in parallel with increasing body weights (181). This growing trend toward larger portions is especially evident in fast food restaurants where "supersizing" of menu items is commonplace (16), and in buffet restaurants where people tend to consume larger portion sizes (180).

2. Physical activity

Three major components of energy expenditure that may influence body weight and composition have been identified: basal metabolic rate (BMR), thermic effect of food and energetic cost of physical activity (182). Thus, most available evidence suggests that low levels of physical activity are associated with an increased risk of obesity (183). The First National Health and Nutrition Examination Survey (NHANES I) carried out between 1971 and 1984 in 8300 individuals showed that low levels of physical activity in the intervening 10-year period were associated with weight gains, and recreational activities were inversely correlated with body weight (184).

Our current environment tends to discourage physical activity (16). Advances in technology and transportation have reduced the need for physical activity in daily life. The appeal of television, electronic games and computers has increased the time spent in sedentary pursuits among children and adults. Facilitating this trend is the fact that most children in the United States do not engage in daily physical activity at school.

Cross-sectional data have often found associations between leisure-time physical activity (inverse) or total amount of time spent sitting down (direct) and BMI (182). Data from the Seven Countries Study indicated that at the population level job-related physical activity is an important determinant of subscapular skinfold thickness (185). In a sample of 4063 children aged 8-16 y from NHANES III (1988-1994), boys and girls who watched ≥ 4h of television/d had the highest skinfold thicknesses and the highest BMIs, highlighting the importance of inactivity (as measured by television viewing) in the etiology of overweight (186). Sedentary lifestyle or physical inactivity is accompanied by a low fat oxidation rate in muscle and a low fat oxidation rate is a risk factor of fat gain or fat re-gain after weight loss (22). Moreover, data from measuring total energy expenditure in Mexican and USA Pima Indians support a significant role for physical activity in the prevention of obesity in genetically susceptible populations (187). Finally, prospective studies provide additional evidence to suggest that a population increase in physical activity may help to prevent the growing prevalence of overweight and obesity over time (188).

3. Interaction of diet and physical activity

Clinical data provide strong evidence that physical activity attenuates the effects of HF diets on positive energy balance. Overall, energy balance was determined not by diet or physical activity alone, but by the interaction between the two (16). Figure 1 shows the hypothetical risk of developing obesity in individuals consuming a high-energy density diet, for example, a typical Western diet. These individuals can maintain a relatively low risk of obesity by engaging in high levels of physical activity, by high dietary restraint, or

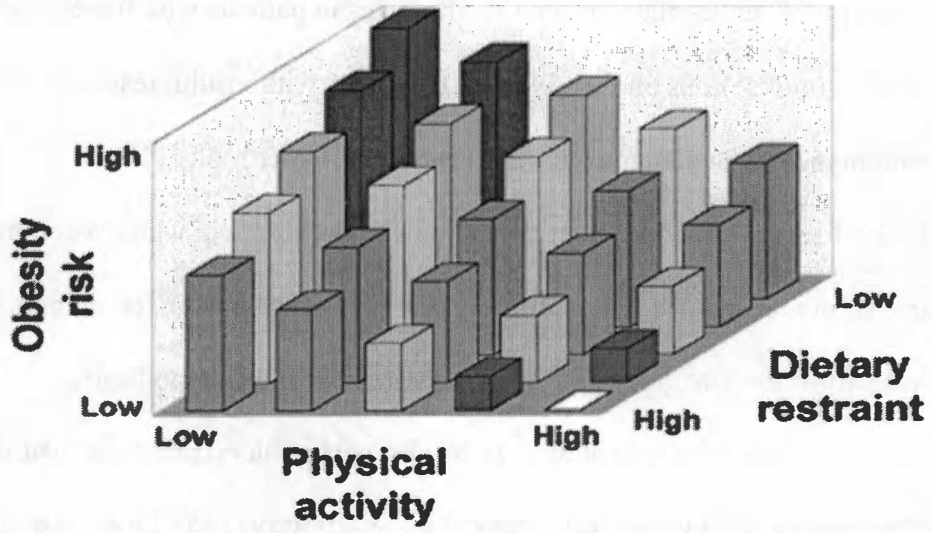

Figure 1. Hypothetical risk of obesity in individuals consuming a diet high in energy density. This risk can be modified by physical activity and by conscious limitation of total energy intake (dietary restraint). As the energy density of the diet decreases, the risk of obesity (the height of all of the bars) would be expected to decline (From Hill 1998, *Science* **280**, 1371)

by a combination of moderate activity with some dietary restraint. For sedentary individuals, a high level of dietary restraint or consumption of a diet low in energy density is required to avoid positive energy balance.

III. Role of Intracellular Ca^{2+} ($[Ca^{2+}]_i$) in Obesity

A. Unifying 'ionic hypothesis'

Obesity is closely related to other metabolic disorders, including insulin resistance/ hyperinsulinemia, hypertension and dyslipidemia, which are integrated into a metabolic syndrome referred to as 'Syndrome X' (189), or the 'deadly quartet' (obesity, hypertriglyceridemia, hypertension and insulin resistance/hyperinsulinemia) (190). Considerable evidence suggests that these conditions are all characterized by an underlying impairment in $[Ca^{2+}]_i$. Sustained elevations of $[Ca^{2+}]_i$ have been observed in

vascular smooth muscle cells, platelets, and erythrocytes in patients with hypertension (191-195), skeletal muscle cells and adipocytes in patients with insulin resistance (196-199), and cardiomyocytes in patients with left ventricular hypertrophy (195). Correspondingly, Resnick proposed a unifying 'ionic hypothesis', in which a common cell lesion underlying these disorders in different tissues was characterized, in part, by elevations in steady-state $[Ca^{2+}]_i$ levels (195). Consistent with this hypothesis, antagonism of Ca^{2+} influx results in clinical improvements in blood pressure, insulin resistance, platelet aggregation, and left ventricular hypertrophy (195). Draznin et al. first reported that obese patients exhibited an elevation in basal adipocyte $[Ca^{2+}]_i$ (196). Further, studies of the mechanisms of agouti-induced obesity indicate that obesity may also be partly a manifestation of a 'lesion' in $[Ca^{2+}]_i$ regulation (63-70).

B. Agouti regulation of adipocyte $[Ca^{2+}]_i$ and lipid metabolism

Agouti was the first obesity gene cloned (51). In addition to the aforementioned central effects of agouti via MC4-R antagonism, peripheral actions of agouti signaling in adipocytes and pancreatic β-cells may also contribute to agouti-induced obesity via modulation of $[Ca^{2+}]_i$ (57).

The carboxyl terminus of agouti protein not only retains full functional activity relative to the intact protein in an *in vitro* assay system (200) but also exhibits a striking structural homology in both number and spacing of cystein residues to the ω-conotoxins and the plectoxins, two toxins produced by the cone snail *Conus geographus* and the hunting spider *Plecteurys tristis*, respectively, that primarily target Ca^{2+} channels (63). This homology between the carboxyl terminus of agouti and ω-conotoxins and the

plectoxins suggests that agouti may act directly upon Ca^{2+} channels and thereby modify channel activity.

Consistent with this, the obese A^y/a yellow mice exhibit increases in both Ca^{2+} influx and steady-state $[Ca^{2+}]_i$ in several tissues (64). This increase in $[Ca^{2+}]_i$ was closely correlated with both the degree of ectopic *agouti* expression and body weight, suggesting the possibility of a causal mechanism between $[Ca^{2+}]_i$ and obesity in these animals. Further, recombinant murine and human agouti proteins caused dose-dependent increases in Ca^{2+} influx and steady-state $[Ca^{2+}]_i$ in a variety of cell types, including both murine and human adipocytes (65). This action of agouti in increasing $[Ca^{2+}]_i$ was inhibited by Ca^{2+} channel antagonism (65) and mimicked by Ca^{2+} channel agonists (69).

Fatty acid synthase (FAS) is a multifunctional enzyme that is highly regulated by nutrients and hormones and is rate-limiting in *de novo* lipogenesis (190). It has been reported that FAS expression and activity are markedly increased in A^y/a mice relative to control mice (66), and that nanomolar concentrations of recombinant agouti protein stimulate ~two-fold increases in FAS gene expression and activity and triglyceride accumulation in 3T3-L1 adipocytes, as well as in human adipocytes, similar to the maximal increases stimulated by insulin (66). This agouti modulation of FAS and lipogenesis is dependent upon Ca^{2+} influx, as it can be mimicked in the absence of agouti via Ca^{2+}-channel activation and inhibited by Ca^{2+}-channel antagonism (66-68).

Indeed, Ca^{2+}-channel blockade has been shown to reduce body weight and fat pad mass effectively in several animal models. Ca^{2+}- channel antagonists, such as nifedipine, verapamil and felodipine, caused decreases in body weight and fat pad mass in obese SHHF/Mcc-fa^{cp} rats (201, 202). Similarly, treatment of transgenic mice ubiquitously

expressing agouti under the control of the β-actin promoter with a Ca^{2+}- channel antagonist (nifedipine) for 4 weeks resulted in significant decreases in adipocyte lipogenesis and an 18% reduction in fat pad mass (203).

The human homologue of agouti, agouti signaling protein (ASIP), is expressed primarily in adipose tissue (56). Previous studies from our laboratory demonstrated that ASIP stimulated lipogenesis in both human and murine adipocytes via a Ca^{2+} -dependent mechanism (66-68, 204). Moreover, ASIP expression was highly correlated with *in vivo* FAS expression and activity in human adipose tissue (204). Thus, ASIP, a physiological Ca^{2+} agonist, may play a role in human obesity, mimicking overexpression of agouti in obese yellow mouse. Interestingly, a recent study involving 8 obese men and 14 obese women showed that ASIP is not only associated with obesity but also gender specific (205). BMI and ASIP gene expression were negatively correlated in men, whereas a positive relationship was observed in women. Hip circumference was significantly higher in women than men. Thus, the sexual dimorphism in the relationship between ASIP and BMI may contribute to the recognized differences in parameters such as hip fat mass distribution between men and women.

In addition to activating lipogenesis, increasing $[Ca^{2+}]_i$ may also contribute to increased triglyceride stores by inhibiting lipolysis. Adipose tissue from obese A^y/a yellow mice exhibited a 50% reduced basal lipolytic rate compared to wild-type littermates (72, 206, 207). The response of this obese adipose tissue to epinephrine (72) and β agonists (206, 207) was also lower than normal. Increasing Ca^{2+} influx with either arginine vasopressin or epidermal growth factor was reported to inhibit lipolysis in rat adipocytes in a Ca^{2+}-dose responsive fashion (208). Further, agouti protein similarly

inhibits both basal and agonist-stimulated lipolysis in human adipocytes via a Ca^{2+}-dependent mechanism. This inhibition can also be mimicked by Ca^{2+} channel agonists and reversed by Ca^{2+} channel antagonists (69). The anti-lipolytic effect of $[Ca^{2+}]_i$ is due to a direct activation of adipocyte phosphodiesterase (PDE) 3B, resulting in a decrease in cAMP and, consequently, reduced ability of agonists to stimulate phosphorylation and activation of hormone-sensitive lipase (HSL) (209). Thus, agouti regulation of adipocyte $[Ca^{2+}]_i$ appears to promote triglyceride storage in human adipocytes by coordinately stimulating lipogenesis and inhibiting lipolysis.

Finally, it is noteworthy that an agouti interaction with insulin is required for the full expression of agouti-induced obesity since aP2-agouti transgenic mice become obese if they are also hyperinsulinemic as a result of either exogenous insulin or a high sucrose diet, while hyperinsulinemia was without effect on body weight in non-transgenic littermate controls (73). Further, hyperplasia of β-cells precedes the development of obesity in A^y/a yellow mice (210), suggesting that hyperinsulinemia may be a direct effect of agouti acting on the pancreas. Since increased $[Ca^{2+}]_i$ is a primary signal for insulin release and agouti increases $[Ca^{2+}]_i$ in several cell types, agouti may stimulate pancreatic Ca^{2+} influx and insulin release as well. Indeed, agouti is expressed and causes a 5-fold increase in Ca^{2+} influx and a 2.2-fold increase in insulin release in human pancreatic islets, as well as in β-cell lines (70), suggesting a potential role for agouti in the development of hyperinsulinemia in humans and in obese A^y/a yellow mice. This hyperinsulinemia may act in an additive or synergistic manner with agouti-stimulated adipocyte Ca^{2+} influx to increase adipocyte triglyceride accumulation (75). In fact, agouti protein and insulin exert independent, additive effects on FAS transcription, with a 6-fold

increase resulting from their combination versus a 2-3-fold increase from each independently (74). FAS responses to agouti are mediated by a distinct agouti/ Ca^{2+} response sequence in the FAS promoter. This sequence maps to the –435 to –415 region of the FAS promoter and is upstream of the insulin response element, which maps to –67 to –52, consistent with the observed additive effects of agouti and insulin on FAS gene transcription (74). Based on these findings, since humans exhibit a similar pattern of adipocyte agouti expression (56), similar agouti/insulin/ Ca^{2+} interactions may result in excessive adipocyte triglyceride storage.

Taken together, these data indicate that regulation of adipocyte $[Ca^{2+}]_i$, possibly coupled with pancreatic $[Ca^{2+}]_i$ and insulin release, may be an important target for the development of therapeutic strategies for the prevention and treatment of obesity, as shown in Figure 2 (57).

C. 1α, 25-$(OH)_2$-D_3 modulation of adipocyte $[Ca^{2+}]_i$ and energy metabolism

Several lines of evidence have suggested a role for 1α, 25-$(OH)_2$-D_3, the biologically active form of the vitamin D, in obesity. Circulating 1α, 25-$(OH)_2$-D_3 levels are elevated in obese individuals (211, 212). Vitamin D receptor (VDR) gene polymorphisms are associated with susceptibility to obesity in patients with early onset type II diabetes (213). Two single nucleotide polymorphisms (SNPs) in nuclear VDR (nVDR) gene intron 8 and exon 9 account for a 9-kg body weight difference, or 4 kg/m^2. Moreover, human body weight and BMI have been shown to be associated with a BsmI restriction site polymorphism in nVDR gene (214). 1α, 25-$(OH)_2$-D_3 induced hyperinsulinemia may also

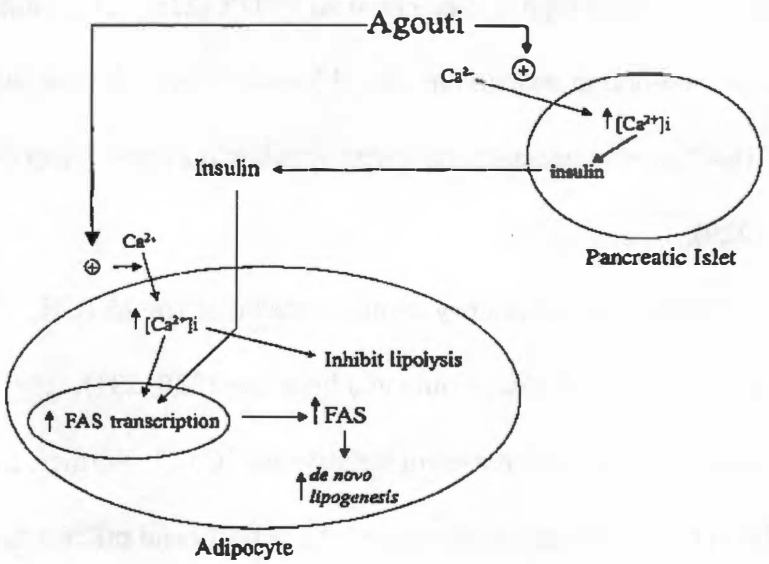

Figure 2. Agouti modulation of adiposity. Agouti stimulates Ca^{2+} influx in pancreatic β-cells, resulting in increased insulin release. Agouti stimulates Ca^{2+} influx in adipocytes, resulting in increased fatty acid synthase (FAS) transcription and activity and inhibition of lipolysis. The agouti-induced insulinemia augments the effects of agouti on adipocytes by independently increasing FAS transcription and inhibiting lipolysis. (From Zemel 2001, CRC press)

contribute to the development of obesity, as 1α, 25-$(OH)_2$-D_3 stimulates insulin secretion (215-217), and VDR gene polymorphism affects insulin secretion and susceptibility to diabetes (218, 219). *In vitro* studies show that 1α, 25-$(OH)_2$-D_3 modulates adipocytes differentiation in several cell models (220-223). Moreover, 1α, 25-$(OH)_2$-D_3 promotes the expression and secretion of lipoprotein lipase in cultured adipocytes (224), indicating that 1α, 25-$(OH)_2$-D_3 may enhance adipocyte lipid accumulation by increasing fatty acid uptake.

It has been previously shown that 1α, 25-$(OH)_2$-D_3 stimulates Ca^{2+} influx in a variety of cells (217, 225-228), including vascular smooth muscle cells (227) and pancreatic β-cells (217) and to play a role in the development of hypertension and hyperinsulinemia, respectively. This effect is rapid and mediated by a putative membrane vitamin D

receptor (mVDR) rather than via the classical nVDR (226, 228). Baran et al. found the plasma membrane-bound protein annexin II to exhibit specific and saturable binding for 1α, 25-$(OH)_2$-D_3, and suggested that it may regulate the rapid effect of 1α, 25-$(OH)_2$-D_3 on $[Ca^{2+}]_i$ (229).

Recent data from our laboratory demonstrated that 1α, 25-$(OH)_2$-D_3 also stimulates Ca^{2+} influx in primary cultures of human adipocytes (230, 231), resulting in significant, sustained dose-responsive increases in steady-state $[Ca^{2+}]_i$. Further, 1α, 25-$(OH)_2$-D_3 caused 50%-100% increases in adipocyte FAS activity and mRNA expression, and an 80% inhibition of lipolysis stimulated by isoproterenol, a β-AR agonist (231). These effects were mimicked by 1α, 25-$(OH)_2$-lumisterol$_3$, a specific agonist for mVDR, and completely blocked by 1β, 25-$(OH)_2$-D_3, a specific antagonist for mVDR (231). Thus, 1α, 25-$(OH)_2$-D_3 modulates adipocyte Ca^{2+} signaling via nongenomic action and, consequently, exerts a coordinated activation of FAS and inhibition of lipolysis, resulting in an expansion of adipocyte triglyceride stores, similar to the action of agouti on adipocytes. More recently, we reported that 1α, 25-$(OH)_2$-D_3 exerted an inhibitory effect on adipocyte UCP2 mRNA and protein levels (232), which may result in decreased thermogenesis and subsequently increased metabolic efficiency. This effect is independently of 1α, 25-$(OH)_2$-D_3-mediated Ca^{2+} influx and the mVDR, and is instead mediated via the nVDR, indicating a novel role of nVDR in regulating adipocyte energy metabolism.

In summary, 1α, 25-$(OH)_2$-D_3-mediated both nongenomic action via Ca^{2+} signal transduction in regulating adipocyte lipid metabolism and genomic action of inhibiting

UCP2 expression via nVDR in regulating metabolic efficiency may represent a suitable target for the development of pharmacological and/or nutritional intervention in obesity.

IV. Role of Dietary Calcium in Modulation of Adiposity

It is now well recognized that dietary calcium plays important roles in the maintenance of bone mass, and in the regulation of hypertension, a major risk factor for cardiovascular disease (233). McCarron, in his evaluation of the relationship between blood pressure and nutrient intake in the U. S population in 1984, first predicted an inverse relationship between dietary calcium and systolic blood pressure (234). Since then, numerous well-controlled studies, especially the DASH (Dietary Approaches to Stop Hypertension) randomized clinical trial, demonstrated that dietary calcium reduces blood pressure (235-237). Notably, the heterogeneity in the blood pressure response to increasing calcium intake was significantly less when dairy sources of calcium as compared to calcium supplements were used in the studies (238). This protective effect of dietary calcium can be explained in large part via suppression of calcitrophic hormones [1α,25-$(OH)_2$-D_3 and/or parathyroid hormone (PTH)] by dietary calcium, thereby normalizing $[Ca^{2+}]_i$ in vascular smooth muscle cells since elevated $[Ca^{2+}]_i$ increased vascular smooth muscle tone, peripheral vascular resistance and blood pressure (239). McCarron also noted a significant inverse relationship between calcium intake and body weight in that same study in 1984 (234). Since then, accumulating animal and human studies now suggest that dietary calcium not only reduces the risk of hypertension and cardiovascular disease, but may play an important role in the prevention and treatment of obesity as well, probably via modulating $[Ca^{2+}]_i$.

A. Animal studies

Metz et al. (240) and Bursey et al. (241) previously reported that increased dietary calcium reduced body weight and body fat composition in spontaneously hypertensive rats (Wistar-Kyoto rats and lean and obese Zucker rats). However, they were unable to demonstrate the mechanism involved in this adiposity modulation. Although some investigators have attributed the effect of dietary calcium on lipid metabolism to inhibition of dietary fat absorption or fecal fat loss, significant fecal energy loss does not result from moderate increases in dietary calcium (242-244).

Multiple studies have shown that increasing dietary calcium suppressed 1α, 25-$(OH)_2$-D_3 levels (195, 245). Moreover, 1α, 25-$(OH)_2$-D_3 stimulates Ca^{2+} influx in adipocytes, thereby promotes triglyceride accumulation by coordinate stimulation of lipogenesis and inhibition of lipolysis (230, 231). Consequently, suppression of 1α, 25-$(OH)_2$-D_3 with high calcium diets would be anticipated to reduce adipocyte $[Ca^{2+}]_i$, stimulate lipolysis, inhibit lipogenesis, thereby exerting an anti-obesity effect.

This concept was first confirmed in aP2-agouti transgenic mice (transgenic mice expressing agouti in adipose tissue under the control of the aP2 promoter, similar to the human pattern of expression) using non-fat dry milk (230). Mice were placed on a low-calcium (0.4%)/high-fat/high-sucrose diet either unsupplemented or with 25 or 50% of the protein replaced by non-fat dry milk or supplemented to 1.2% Ca with calcium carbonate for 6 weeks. At the end of the study, mice on low calcium (0.4%) diet exhibited marked increases in adipocyte lipogenesis, inhibited lipolysis, and accelerated increases in body weight and adipose tissue mass. However, all three high calcium (1.2%) diets reduced FAS expression and activity by 51% and stimulated lipolysis 3 to 5-

fold, resulting in 26 to 39% reductions in body weight and adipose tissue mass, respectively. The magnitude of these effects depended upon the source of dietary calcium, with dairy sources of calcium exerting significantly greater effects than calcium carbonate. Thus, increasing dietary calcium modulates adipocyte lipid metabolism by coordinatedly down-regulating lipogenesis and up-regulating lipolysis, and attenuates diet-induced obesity risk.

A follow-up study was conducted to determine whether increasing dietary calcium would accelerate fat loss secondary to caloric restriction following dietary induction of obesity (246). Administration of the same low-calcium(0.4%)/high-fat/high-sucrose diet to aP2-agouti transgenic mice for 6 weeks resulted in a ~100% increase in adipocyte $[Ca^{2+}]_i$ and a corresponding weight gain of 29% and a 2-fold increase in total fat pad mass, demonstrating that elevation of adipocyte $[Ca^{2+}]_i$ is associated with increased adiposity in aP2-agouti transgenic mice. The animals were then placed on energy restriction (70% of an *ad libitum* fed control group) for an additional 6 weeks. Energy restriction on the low calcium diet (0.4%) failed to reduce $[Ca^{2+}]_i$ and only reduced body weight and fat pad mass by 11% and 8%, respectively. In contrast, energy restriction in conjunction with high calcium (1.2%) diets normalized $[Ca^{2+}]_i$ and resulted in 19 to 29% reductions in body weight and 42 to 69% decreases in fat pad mass depending on calcium sources (elemental vs. dairy). Interestingly, the animals on the low calcium diets were unable to increase adipocyte lipolysis or suppress lipogenesis despite being on an energy-restricted regimen. In contrast, the high calcium diets caused marked reductions in FAS expression and activity (35 to 81%), 2 to 3-fold increases in lipolysis during energy restriction. In addition, *ad lib* feeding of the basal low-calcium diet caused sustained

hyperinsulinemia in the aP2-agouti transgenic mice. Energy restriction per se reduced the plasma insulin levels, but increasing dietary calcium did not further reduce insulin levels but nonetheless exerted potent effects on body weight and fat pad mass reduction. This indicates that the effects of dietary calcium on attenuating weight gain and accelerating weight loss are not due to insulin suppression and instead are likely to result from the observed suppression of adipocyte $[Ca^{2+}]_i$ by suppressing 1α, 25-$(OH)_2$-D_3 and subsequent modulation of adipocyte lipid metabolism. Notably, dairy calcium (non-fat dry milk) exerts approximately twice the effect of the calcium supplement on both adipocyte lipid metabolism and on body fat and body weight despite that all three high calcium diets exerted comparable effects in lowering $[Ca^{2+}]_i$. This finding suggests that other components of dairy may act via a Ca^{2+}-independent system to further reduce adiposity.

In this study, it was also found that mice fed high calcium diets exhibited higher core temperature (0.48 to 0.67°C) and increased expression of white adipose tissue UCP2. UCP2 has been shown to stimulate mitochondrial proton leak and therefore play a potential role in thermogenesis, metabolic efficiency and obesity (102, 103). Core temperature is an indirect metabolic index associated with thermogenesis. In fact, an increased core temperature was observed in another study in transgenic mice ubiquitously expressing agouti under the control of the β-actin promoter after mice were treated with Ca^{2+} channel antagonist nifedipine (203). The contribution of thermogenesis to anti-obesity action of Ca^{2+} channel blockade has been addressed in previous studies (247, 248). However, this mechanism remains controversial in that some investigators have

attributed the thermogenic effect of Ca^{2+} channel blockade to the increased function of BAT. This study of dietary calcium modulation of adiposity suggests that up-regulation of UCP2 expression may be responsible for the increased thermogenesis. This concept has been confirmed by our recent study demonstrating that 1α, 25-$(OH)_2$-D_3 exerted an inhibitory effect on adipocyte UCP2 mRNA and protein levels via the nVDR, independent of its effects on Ca^{2+} influx via the mVDR (232). Thus, suppression of 1α, 25-$(OH)_2$-D_3 with high calcium diets and consequent up-regulation of UCP2 via a genomic action may contribute to our observations of increased thermogenesis in mice fed with high calcium diets. This effect, coupled with decreased lipogenesis and increased lipolysis secondary to decreased $[Ca^{2+}]_i$ mediated by nongenomic action may contribute to an anti-obesity effect of dietary calcium as shown in Figure 3 (233).

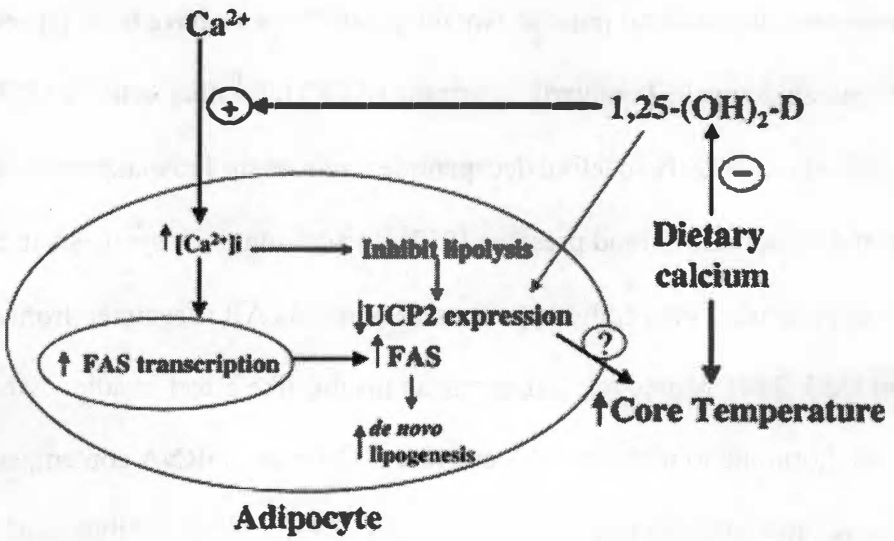

Figure 3. Mechanisms of the anti-obesity effect of dietary calcium (From Zemel 2002, J. Am. Coll. Nutr. 21, 146S)

This concept of dietary calcium modulation of adiposity was further extended in aP2 agouti-transgenic mice by using a calcium-fortified breakfast cereal as a calcium source to increase dietary calcium from 0.4% to 1.2% (249). The effects of the high calcium cereal were similar to those described above for calcium carbonate, producing significant attenuation of diet-induced obesity and acceleration of weight/fat loss during caloric restriction. Interestingly, the addition of a small amount of non-fat dry milk, sufficient to increase dietary calcium from 1.2% to 1.3% (with dietary macronutrients held constant) doubled the rate of fat loss. Thus, dairy sources of calcium exert a greater anti-obesity effect than calcium carbonate. Although the additional factor(s) in dairy responsible for this effect have not yet been identified, milk is recognized as a rich source of bioactive compounds (250), which may act independently or synergistically with the calcium to attenuate lipogenesis, accelerate lipolysis and/or affect nutrient partitioning between adipose tissue and skeletal muscle. Notably, milk proteins have been reported to contain significant angiotensin-I converting enzyme (ACE) inhibitory activity (251, 252). The ACE hydrolyses largely inactive decapeptide angiotensin I to octapeptide angiotensin II (AII), which increases blood pressure (252). In addition to its synthesis in the classical rennin angiotensin system, the hypertensive hormone AII is secreted from adipose tissue as well (253, 254). Moreover, AII exerts an insulin-like effect in adipocytes and acts as a lipogenic hormone to increase FAS enzyme activity and mRNA content, subsequently increasing fatty acid and triglyceride synthesis (255). Indeed, inhibition of the rennin-angiotensin system mildly attenuates obesity in rodents, and limited clinical observations support this concept in hypertensive patients treated with ACE inhibitors (256).

Collectively, the primary effect of dietary calcium appears to be inhibition of 1α,25-$(OH)_2$-D_3 effects on energy storage and utilization. Adipocyte Ca^{2+} signaling is an attractive target for the development of obesity interventions (57, 233, 239).

B. Human studies

Since McCarron first noted a significant inverse relationship between calcium intake and body weight in his evaluation of the relationship between blood pressure and nutrient intake in the U. S population in 1984 (234), several lines of evidence from human studies have supported this relationship although the mechanism was not explored at that time. Data from the Nationwide Food Consumption Survey (1987-88) demonstrated that individuals with the lowest calcium intakes tended to have the highest body weights. Moreover, African-Americans consume the lowest level of calcium and exhibit the greatest prevalence of obesity (257). During the course of a clinical trial investigating the antihypertensive effect of calcium in obese African-Americans, it was noted that 12 months of yogurt supplementation, sufficient to raise daily calcium intake from approximately 400 to 1000 mg/day, resulted in a 4.9 kg reduction in body fat in obese African-Americans without an accompanying reduction in caloric intake (230).

A randomized clinical trial demonstrated a markedly greater weight loss (7.0 vs. 1.7 kg) in patients maintained on a milk-based diet for 16 wk, compared with those maintained on conventional hypocaloric diet at the same level of energy intake (258). Although this difference was attributed to the novelty of the milk-based diet possibly contributing to a greater level of compliance, our data suggest that this effect may also be

attributed to suppression of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ and adipocyte $[Ca^{2+}]_i$ by dairy calcium, with a consequent reduction in the efficiency of energy utilization.

Recently, accumulating human studies strongly support the anti-obesity effect of dietary calcium. In a two-year prospective study of 54 normal weight young women aged 18 to 31 years participating in an exercise intervention (259), subjects with high calcium intake, corrected by total energy intake, gained less body weight and body fat than those with low calcium intake. Moreover, increased total calcium and dairy calcium intakes predicted fat mass reductions independently of caloric intake for women at lower energy intakes (below the mean of 1,876 kcal/d). In Phase 2 of the Quebec Family Study, adults between 20 and 65 years of age (235 men and 235 women) were divided into three groups according to their daily calcium intake as follows: group A<600mg, group B between 600 and 1000 mg and group C>1000mg. It was found that a low daily calcium intake is associated with an increase in body fat, particularly in women (260).

Davis et al. (261) have conducted a series of calcium intervention studies designed with the bone mass the original outcome variable, and have recently re-evaluated these data with body weight and body fat the outcome variable. The re-analysis involved 780 women who participated in five clinical trials (i.e., four observational and one double-blind, placebo-controlled randomized trial). Analysis reveals a consistent negative associations between calcium intake and body weight for all age groups (3rd, 5th and 8th decades of life), and an odds ratio for being overweight of 2.25 for young women in the lower half versus the upper half of calcium intake. Data from the randomized controlled trial demonstrated a calcium treatment effect of 0.325 kg weight loss per year over four years with no intentional change in caloric intake; overall, the relationships derived from

this re-analysis indicate that a calcium intake increase of 1000 mg/day is associated with an 8 kg reduction in body weight.

A similar beneficial effect of dietary calcium on body fat mass accumulation has been demonstrated in growing children as well. In one short-term study (262), 50 healthy non-obese children with low calcium intakes (<800mg daily) aged 2.5 to 8.8 years were studied for 6 months. The children were randomly divided into a dairy or control group. The dairy group was supplemented to 1200 mg calcium or 4 servings of dairy foods daily. Control children ingested their usual diet with calcium intake less than 800 mg daily. At the end of 6 months, children from the dairy group gained less body fat than those from the control group. Similarly, in another five-year longitudinal study of preschool children (263), higher mean longitudinal calcium intakes and more servings/day of dairy products were associated with lower body fat.

To extend those findings of dietary calcium modulation of adiposity in aP2-agouti transgenic mice to humans, we recently conducted a clinical trial involving 32 obese adults (264). They were maintained for 24 weeks on balanced 500 kcal/day deficit diets and randomized to control group (400-500mg Ca/day), high calcium group (control diet supplemented with 800 mg Ca/day), or high dairy group (3-4 servings of low-fat dairy products/day, total Ca intake of 1200-1300 mg/day). At the end of the study period, patients from the control group lost only 6.4% of their body weight while those from the high calcium group and high dairy group lost 26% and 70% more, respectively. Fat loss followed a similar trend, with the high calcium and high dairy diets augmenting the fat loss found on the low calcium diet by 38% and 64%, respectively. Moreover, fat loss from the trunk region represented 19% of total fat loss on the low calcium diet, and high

calcium and high dairy diets increased this number to 50% and 66%, respectively. Thus, increasing dietary calcium significantly augments weight and fat loss secondary to caloric restriction and increases the percentage of fat lost from the trunk region. Moreover, dairy products exert a substantially greater effect on both fat loss and fat distribution compared to an equivalent amount of supplemental calcium.

Finally, at the population level, a role for dietary calcium in modulating body composition was assessed via analysis of the third National Health and Nutrition Examination Survey (NHANES III) (230). After controlling for energy intake, age, race/ethnicity and activity level, the odds ratio of being in the highest quartile of percent body fat was reduced from 1.0 for the first quartile of calcium intake to 0.75, 0.40 and 0.16 for the second, third and fourth quartiles of calcium intake, respectively, for women; a similar inverse relationship was also noted in men. These data indicate that for any given level of energy intake and expenditure, a low calcium diet favors increased adipose tissue energy storage while a higher calcium diet favors increased energy utilization.

In summary, laboratory, clinical and population data all support a significant anti-obesity effect of dietary calcium, and dairy sources of calcium seem to exert greater effects than calcium supplement. Therefore, dietary calcium could be an effective regimen for the prevention and treatment of obesity at the population level.

LITERATURE CITED

1. Campfield, L. A., and Smith, F. J. (1999) The pathogenesis of obesity. *Best Prac. Res. Clin. Endocrinol. Metab.* **13** (1), 13-30

2. Royal College of Physicians (1983) Obesity. *J. R. Coll. Physicians. Lond.* **17**, 3-58

3. Manson, J. E., Willett, W. C., and Stampfer, M. J. (1995) Body weight and mortality among women. *N. Engl. J. Med.* **103**, 1024-1029

4. National Institute of Health and National Heart, Lung, and Blood Institute (1998) Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: The evidence report.

5. Flegal, K. M., Carroll, M. D., Kuczmarski, R. J., and Johnson, C. L. (1998) Overweight and obesity in the United States: prevalence and trends, 1960-1994. *Int. J. Obes. Relat. Metab. Disord.* **22**, 39-47

6. World Health Organization (1998) *Obesity: Preventing and Managing the Global Epidemic* (World Health Organization, Geneva)

7. Flegal, K. M., Carroll, M. D., Ogden, C. L., amd Johnson, C. L. (2002) Prevalence and trends in obesity among US adults, 1999-2000. *JAMA* **288**, 1723-1727

8. Foreyt, J. P., and Goodrick, G. K. (1995) The ultimate triumph of obesity. *Lancet* **346**, 134-135

9. Ogden, C. L., Flegal,K. M., Carroll, M., and Johnson, C. L. (2002) Prevalence and trends in overweight among U.S. children and adolescents, 1999-2000. *JAMA* **288**, 1728-1732

10. Goran, M. I. (2001) Metabolic precursors and effects of obesity in children: a decade of progress, 1990-1999. *Am. J. Clin. Nutr.* **73**, 158-171

11. World Health Organization (2000) Obesity: preventing and managing the global epidemic. Report of a WHO consultation. *World Health Organ. Tech. Rep. Ser.* **894**, i-xii, 1-253

12. Pinhas-Hamiel, O., Dolan, L. M., Daniels, S. R., Standiford, D., Khoury, P. R., and Zeitler, P. (1996) Increased incidence of non-insulin-dependence diabetes mellitus among adolescents. *J. Pediatr.* **128**, 608-615

13. Allison, D. B. Fontaine, K. R., Manson, J. E., Stevens, J., and VanItallie, T. B. (1999) Annual deaths attributable to obesity in the United States. *JAMA* **282,** 1530-1538

14. Wolf, A. M., and Colditz, G. A. (1996) Social and economic effects of body weight in the United States. *Am. J. Clin. Nutr.* **63** (Suppl. 3), 466S-469S

15. Bouchard, C., and Tremblay, A. (1997) Genetic influences on the response of body fat and fat distribution to positive and negative energy balances in human identical twins. *J. Nutr.* **127** (Suppl. 5), 943S-947S

16. Hill, J. O., and Peters, J. C. (1998) Environmental contributions to the obesity epidemic. *Science* **280,** 1371-1374

17. Wickelgren, I. (1998) Obesity: How big a problem? *Science* **280,** 1364-1367

18. Goldstein, D. J. (1992) Beneficial effects of modest weight loss. *Intl. J. Obes.* **16,** 397-415

19. Blackburn, G. (1995) Effect of degree of weight loss on health benefits. *Obes. Res.* **3** (Suppl. 2), 211S-218S

20. Williamson, D. F. (1995) Prospective study of intentional weight loss and mortality in never-smoking overweight US white women aged 40-64 years. *Am. J. Epidem.* **141,** 1128-1141

21. Martinez, J. A. (2000) Obesity in young Europeans: genetic and environmental influences. *Eur. J. Clin. Nutr.* **54** (Suppl. 1), S56-60

22. Maffeis, C. (2000) Etiology of overweight and obesity in children and adolescents. *Eur. J. Pediatr.* **159** (Suppl. 1), S35-44

23. Comuzzie, A. G., and Allison, D. B. (1998) The search for human obesity genes. *Science* **280,** 1374-1377

24. Froguel, P., and Boutin, P. (2001) Genetics of pathways regulating body weight in the development of obesity in humans. *Exp. Bio. Med.* **226** (11), 991-996.

25. Naggert, J., Harris, T., and North, M. (1997) The genetics of obesity. *Curr. Opin. Gene. Dev.* **7,** 398-404.

26. Bray, G. A. (1997) Progress in understanding the genetics of obesity. *J. Nutr.* **127,** 940S-942S.

27. Ingalls, A. M., Dickie, M. M., and Snell, G. D. (1953) Obesity, a new mutation in the house mouse. *J. Hered.* **41**, 317-318

28. Coleman, D. L. (1978) Obese and diabetes: Two mutant genes causing diabetes-obesity syndromes in mice (review). *Diabetologia* **14**, 141-148

29. Zhang, Y. Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425-432

30. Chen, H., Charlat, O., Tartaglia, L. A., Woolf, E. A., Weng, X., Ellis, S. J., Lakery, N. D., Culpepper, J., Moore, K. J., Breitbart, R. E., Duyk, G. M., Tepper, R. I., and Morgenstern, J. P. (1996) Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* **84**, 491-495

31. Lee, G. H., Proenca, R., Montez, J. M., Carroll, K. M., Darvishzadeh, J. G., Lee, J. I., and Friedman, J. M. (1996) Abnormal splicing of the leptin receptor in diabetic mice. *Nature* (Lond.) **379**, 632-635

32. Tartaglia, L. A., Bembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., and Deeds, J. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**, 1263-1271

33. Tartaglia, L. A. (1997) The leptin receptor. *J. Biol. Chem.* **272**, 6093-6096

34. Banks, W. A., Kastin, A. J., Huang, W., Jaspan, J. B., and Maness, L. M. (1996) Leptin enters the brain by a saturable system independent of insulin. *Peptides* **17**, 305-311

35. Chua, S. C., Jr., Chung, W. K., Wu-Peng, X. S., Zhang, Y., Liu, S. M., Tartaglia, L., and Leibel, R. L. (1996) Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* **271**, 994-996

36. Phillips, M. S., Liu, Q., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. J., and Hess, J. F. (1996) Leptin receptor missense mutation in the fatty Zucker rat. *Nat. Genet.* **13**, 18-19

37. Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995) Effects of the obese gene-product on body-weight regulation in OB/OB mice. *Science* **269**, 540-543

38. Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowi, D., Lallone, R. L., Burley, S. K., and Friedman, J. M. (1995) Weight-reducing effects of the plasma-protein encoded by the obese gene. *Science* **269**, 543-546

39. Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R., and Burn, P (1995) Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* **269**, 546-549

40. Chehab, F. E., Lim, M. E., and Lu, R. H. (1996) Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat. Genet.* **12**, 318-320

41. Friedman, J. M., and Halaas, J. L. (1998) Leptin and the regulatin of body weight in mammals. *Nature* **395**, 763-770

42. Schwartz, M. W., Woods, S. C., Porte, D., Seeley, R. J., and Baskin, D. G. (2000) Central nervous system control of food intake. *Nature* **404**, 661-671

43. Stephens, T. W., Basinski, M., Bristow, P. K., Bue-Valleskey, J. M., Burgett, S. G., Craft, L., hale, J., Hoffmann, J., Hsiung, H. M., and kriauciunas, A. (1995) The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* (Lond.) **377**, 530-532

44. York, D. A., and Hansen, B. (1997) Animal models of obesity. Handbook of obesity. Marcel Dekker, Inc. 191-221

45. Hamilton, B. S., Paglia, D., Kwan, A. Y., and Deitel, M. (1995) Increased obese mRNA expression in omental fat cells from massively obese humans. *Nat. Med.* **1**, 953-956

46. Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., Fei, H., Kim, S., Lallone, R., and Ranganathan, S. (1995) Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight reduced subjects. *Nat. Med.* **1**, 1155-1161

47. Schwartz, M. W., Peskind, E., Raskind, M., Boyko, E. J., and Porte, D. Jr. (1996) Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. *Nat. Med.* **2**, 589-593

48. Considine, R. V., Considine, E. L., Williams, C. J., Hyde, T. M., and Caro, J. F. (1996) The hypothalamic leptin receptor in humans. Identification of incidental sequence polymorphisms and absence of the *db/db* mouse and *fa/fa* rat mutations. *Diabetes* **45**, 992-994

49. Yen, T. T., Gill, A. M., Frigeri, L. G., Barsh, G. S., and Wolff, G. L. (1994) Obesity, diabetes, and neoplasia in yellow A^{vy}/- mice: ectopic expression of the agouti gene. *FASEB. J.* **8**, 479-488

50. Michauld, E. J., Mynatt, R. L., Miltenberger, R. J., Klebig, M. L., Wilkison, J. E., Zemel, M. B., Wilkison, W. O., and Woychik, R. P. (1997) Role of the agouti gene in obesity. *J. Endoncrinol.* **155**, 207-209

51. Bultman, S. J., Michaud, E. J., and Woychik, R. P. (1992) Molecular characterization of the mouse agouti locus. *Cell* **71**, 1195-1204

52. Miller, M. W, Duhl, D. M.J., Vrieling, H., Cordes, S. P., Ollmann, M. M., Winkes, B. M., and Barsh, G. S. (1993) Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. *Genes Dev.* **7**, 454-462

53. Galbraith, D. B. (1964) The agouti pigment pattern of the mouse: a quantitative and experimental study. *J. Exp. Zool.* **155**: 71-76

54. Perry, W., Hustad, C., Swing, D., Jenkins, N., and Copeland, N. (1995) A transgenic mouse assay for agouti protein activity. *Genetics* **140**, 267-274

55. Klebig, M. L., Wilkinson, J. E., Geisler, J. G., and Woychik, R. P. (1995) Ectopic expression of the agouti gene in transgenic mice causes obesity, features of type II diabetes and yellow fur. *Proc. Natl. Acad. Sci. USA* **92**, 4728-4732

56. Kwon, H. Y., Bultman, S. J., Loffler, C., Chen, W. J., Furdon, P. J., Powell, J. G., Usala, A. L., Wilkison, W., Hansmann, I., and Woychik, R. P. (1994) Molecular structure and chrosomal mapping of the human homolog of the mouse agouti gene. *Proc. Natl. Acad. Sci. USA* **91**, 9760-9768

57. Zemel, M. B., Xue, B. Z., and Shi, Hang (2001) The agouti gene in obesity: Central and peripheral mechanisms, and therapeutic implications in Nutrient-gene interactions in health and disease, Eds, Moustaid-Moussa, N. & Berdanier, C. D., CRC series in Modern Nutrition, CRC press

58. Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S. J., DelValle, J., and Yamada, Y. (1993) Molecular cloning , expression, and gene localization of a fourth melanocortin receptor. *J. Bio. Chem.* **268**, 15174-15180

59. Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., and Wilkison, W. O. (1994) Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* **371**, 799-802

60. Mountjoy, K. G., Mortrud, M. T., Low, M. J., Simerly, R. B., and Cone, R. D. (1994) Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol.* **8**, 1298-1308

61. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., and Cone, R. D. (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* **385**, 165-168

62. Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J., Fang, Q., Berkemeier, L. R., Gu, W., kesterson, R. A., Boston, B. A., and Cone, R. D. (1997) Targeted distruction of the melanocortin-4 receptor results in obesity in mice. *Cell* **88**, 131-141

63. Manne, J., Argeson, A. C., and Siracusa, L. D. (1995) Mechanisms for the pleiotropic effects of the agouti gene. *Proc. Natl. Acad. Sci. USA* **92**, 4721-4724

64. Zemel, M. B., Kim, J. H., Woychik, R. P., Michaud, E. J., Kadwell, S. H., Patel, I. R., and Wilkison, W. O. (1995) Agouti regulation of intracellular calcium: Role in the insulin resistance of viable yellow mice. *Proc. Natl. Acad. Sci. USA* **92**, 4733-4737

65. Kim, J. H., kieffer, L. L., Woychik, R. P., Wilkison, W. O., Truesdale, A., Ittoop, O., Willard, D., Nichols, J., and Zemel, M. B. (1997) Agouti regulation of intracellular calcium. Role of melanocortin receptor. *Am. J. Physiol.* **272**, E 379-E385

66. Jones, B. H., Kim, J. H., Zemel, M. B., Woychik, R. P., Michaud, E. J., Wilkison, W. O., and Moustaid, N. (1996) Upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. *Am. J. Physiol.* **270**, E192-E199

67. Zemel, M. B., Kim, J. H., Jones, B. H., Moore, J. W., Woychik, R. P., Moustaid, N., and Wilkison, W.O. (1995) Agouti gene product regulation of intracellular free calcium results in stimulation of fatty acid synthase. *Obes. Res.* **3**, 338S

68. Moore, J. W., Willard, D., Moustaid, N., Wilkison, W. O., and Zemel, M. B. (1996) Role of intracellular calcium in agouti and insulin modulation of fatty acid synthase. *FASEB J.* **10**, A187 (Abstr.)

69. Xue, B., Moustaid-Moussa, N., Wilkison, W. O., and Zemel, M. B. (1998) The agouti gene products inhibits lipolysis in human adipocytes via a Ca^{2+} dependent mechanism. *FASEB J.* **12**, 1391-1396

70. Xue, B. Z., Wilkison, W. O., Mynatt, R. L., Moustaid, N., Goldman, M., and Zemel, M. B. (1999) The agouti gene products stimulates pancreatic β-cell Ca^{2+} signaling and insulin release. *Physiol. Genomics* **1**, 11-19

71. Yen, T. T., Greenberg, M. M., Yu, P. L., and Pearson, D. V. (1976) An analysis of the relationships among obesity, plasma insulin and hepatic lipogenic enzymes in "viable yellow obese" mice (A^{vy} /a). *Horm. Metab. Res.* **8**, 159-167

72. Yen, T. T., Steinmetz, J., and Wolff, G. L. (1970) Lipolysis in genetically obese and diabetes-prone mice. *Horm. Metab. Res.* **2**, 200-207

73. Mynatt, R. L., Miltenberger, R. J., Klebig, M. L., Zemel, M. B., Wilkinson, J. E., Wilkison, W. O., and Woychik, R. P. (1997) Combined effects of insulin treatment and adipose tissue-specific agouti expression on the development of obesity. *Proc. Natl. Acad. Sci. USA* **94**, 919-922

74. Claycombe, K. J., Wang, Y., Jones, B. H., Kim, S., Wilkison, W. O., Zemel, M. B., Chun, J., and Moustaid-Moussa, N. (1997) Transcriptional regulation of the adipocyte fatty acid synthase gene by the agouti gene product: Interaction with insulin. *FASEB J.* **11**, A352 (Abstr.)

75. Zemel, M. B., Nynatt, R. L., and Dibling, D. (1999) Synergism between diet-induced hyperinsulinemia and adipocyte-specific agouti expression. *FASEB J.* **13**, A873 (Abstr.)

76. Coleman, D. L., and Eicher, E. M. (1990) Fat (*fat*) and tubby (*tub*): two autosomal recessive mutations causing obesity syndromes in the mouse. *J. Hered.* **88**, 424-427

77. Naggert, J. K., Fricher, L. D., Varlamov, O., Nishina, P. M., Rouille, Y., Steiner, D. F., Carroll, R. J., Paigen, B. J., and Leiter, E. H. (1995) Hyperproinsulinemia in obese fat/fat mice associated with a carboxypeptidase E mutation which reduces enzyme activity. *Nat. Genet.* **10**, 135-142

78. Cool, D. R., Normant, E., Shen, F., Chen, H., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) Carboxypeptidase E is a regulated secretory pathway sorting receptor: genetic obliteration leads to endocrine disorders in Cpe/fat mice. *Cell* **88**, 73-83

79. Rovere, C., Viale, A., Nahon, J., and Kitabgi, P. (1996) Impaired processing of brain proneurotensin and promelanin-concentrating hormone in obese *fat/fat* mice. *Endocrinology* **137**, 2954-2958

80. Noben-Trauth, K., Naggert, J. K., North, M. A., and Nishina, P. M. (1996) A candidate gene for the mouse mutation tubby. *Nature* **380**, 534-538

81. Kleyn, P. W., Fan, W., Kovats, S. G., Lee, J. J., Pulido, J. C., Wu, Y., Berkemeier, L. R., Misumi, D. J., Holmgren, L., and Charlat, O. (1996) Identification and characterization of the mouse obesity gene *tubby*: a member of a novel gene family. *Cell* **85**, 281-290

82. Santagata, S., Boggon, T. J., Baird, C. L., Gomez, C. A., Zhao, J., Shan, W. S., Myszka, D. G., and Shapiro, L. (2000) G-protein signaling through tubby proteins. *Science* **292**, 2041-2050

83. Bray, G. A. (1991) Obesity, a disorder of nutrient partitioning: the MONA LISA hypothesis. *J. Nutr.* **121**, 1146-1162

84. Stenzel-Poore, M. P., Caneron, V. A., Vaughan, J., Sawchenko, P. E., and Vale, W. (1992) Development of Cushing's Syndrome in Corticotropin-Releasing Factor Transgenic Mice. *Endocrinology* **130**, 3378-3386

85. Pepin, M.-C., Pothier, F., and Barden, N. (1992) Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene. *Nature* **355**, 725-728

86. Fletcher, J. M. (1986) Effects of adrenalectomy before weaning in the genetically obese Zucker rat (fa/fa). *Br. J. Nutr.* **56**, 141-151

87. Satio, M., and Bray, G. A (1984) Adrenalectomy and food restriction in the genetically obese (ob/ob) mouse. *Am. J. Physiol.* **246**, R20-R25

88. Shimomura, Y., Bray, G. A., and Lee, M. (1987) Adrenalectomy and steroid treatment in obese (ob/ob) and diabetic (db/db) mice. *Horm. Metab. Res.* **19**, 295-299

89. Shimizu, H., Shargill, N. S., Bray, G. A. (1989) Adrenalectomy and response to corticosterone and MSH in the genetically obese yellow mouse. *Am. J. Physiol.* **256**, R494-R500

90. Kahn, B. B. (1992) Facilitative glucose transporters: Regulatory mechanisms and dysregulation in Diabetes. *J. Clin. Invest.* **89**, 1367-1374

91. Shepherd, P. R., Gnudi, L. Tozzo, E., Yang, H., Leach, F., and Kahn, B. B. (1993) Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT 4 selectively in adipose tissue. *J. Bio. Chem.* **268**, 22243-22246

92. Yaswen, L. et al. (1999) Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat. Med.* **5**, 1066-1070

93. Krude, H., Biebermann, H., Luck, W., Horn, R., Brabant, G., and Gruters, A. (1998) Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in human. *Nature Genet.* **19**, 155-157

94. Nicholls, D. G., and Locke, R. M. (1984) Thermogenic mechanisms in brown fat. *Physiol. Rev.* **64**, 1-64

95. Himms-Hagen, J. (1990) Brown adipose tissue thermogenesis: interdisciplinary studies. *FASEB J.* **4**, 2890-2898

96. Lowell, B. B., Susulic, V. S., Hanann, A., Lawitts, J. A., Himms-Hagen, J., Boyers, B. B., Kozak, L. P., and Flier, J. S. (1993) Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* **366**, 740-742

97. Melnyk, A., Harper, M.-E., and Himms-Hagen, J. (1997) Raising at thermoneutrality prevents obesity and hyperphagia in brown adipose tissue-ablated transgenic mice. *Am. J. Physiol.* 272, R1088-R1093

98. Sakaguchi, T., Takahashi, M., and Bray, G. A. (1988) Diurnal changes in sympathetic activity: relation to food intake and to insulin injected into the ventromedial or suprachiasmatic nucleus. *J. Clin. Invest.* **32**, 282-286

99. Bray, G. A. (1991) Reciprocal relation between the sympathetic nervous system and food intake. *Brain Res. Bull.* **27**, 517-520

100. Himms-Hagen, J. (1995) Role of BAT thermogenesis in control of thermoregulatory feeding in rats. A new hypothesis that links thermostatic and glucostatic hypotheses for control of food intake. *Pro. Soc. Exp. Biol. Med.* **208**, 159-169

101. Enerback, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Harper, M.-E., and Kozak, L. P. (1997) Mice lacking mitochondria uncoupling protein are cold-sensitive but not obese. *Nature* **387**, 90-94

102. Fleury, C., Neverova, M., Collins, S., Raimbaut, S., Champigny, O., Meyruies, C. L., Bouillaud, F., Seldin, M., Surwit, R. S., Ricquier, D., and Warden, G. H. (1997) Uncoupling protein 2: a novel gene linked to obesity and hyperinsulinemia. *Nature Gen.* **15**, 269-272

103. Gimeno, R. E., Dembski, M., Weng, X., Deng, N., Shyjan, A. W., Gimeno, C. J., Iris, F., Ellis, S. J., Woolf, E. A., and Tartaglia, L. A. (1997) Cloning and characterization of an uncoupling protein homolog. *Diabetes* **46**, 900-906

104. Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A. G., Seydoux, J., Muzzin, P., and Giacobino, J. P. (1997) Uncoupling protein-3: a new member of the mitochondria carrier family with tissue specific expression. *FEBS Lett.* **408**, 39-42

105. Vidal-Puig, A., Solanes, G., Grujic, D., Flier, J., S., and Lowell, B. B. (1997) UCP3: an uncoupling protein homolog expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem. Biophy. Res. Commun.* **235**, 79-82

106. Hinz, W., Faller, B., Gruninger, S., Gazzotli, P., and Chiesi, M. (1999) Recombinant human uncoupling protein-3 increases thermogenesis in yeast cells. *FEBS Lett.* **448**, 57-61

107. Clapman, J. C., Arch, J., Chapman, H., Harper, A. J., Brand, M, D., and Abuin, A. (2000) Mice overexpressing human uncoupling proein-3 in skeletal muscle are hyperphagic and lean. *Nature* **406**, 415-418

108. Vidal-Puig, A. J., Grujic, D., Zhang, C., Hagen, T., Boss, O., Ido, Y., Szczeponik, A., Wade, J., Mootha, V., Cortright, R., Muoio, D. M., and Lowell, B. B. (2000) Energy metabolism in uncoupling protein-3 gene knockout mice. *J. Bio. Chem.* **275**, 16258-16266

109. Rolfe, D. F., and Brand, M. D. (1996) Contribution of mitochondria proton leak to skeletal muscle respiration and to standard metabolic rate. *Am. J. Physiol.* **271**, C1380-C1389

110. Samec, S., Seydoux, J., and Dulloo, A. G. (1998) Role of UCP homologs in skeletal muscles and brown adipose tissue: mediators of thermogenesis or regulators of lipids as fuel substrate? *FASEB J.* **12**, 715-724

111. Samec, S., Seydoux, J., and Dulloo, A. G. (1998) Interorgan signaling between adipose tissue metabolism and skeletal muscle uncoupling protein homologs. Is there a role for circulating free fatty acids? *Diabetes* **47**, 1693-1698

112. Bodrova, M. E., Dedukhova, V. I., Mokhova, E. N., and Skulachev, V. P. (1998) Membrane potential generation coupled to oxidation of external NADH in liver mitochondria. *FEBS Lett.* **435**, 269-274

113. Papa, S., and Skulochev, V. P. (1997) Reactive oxygen species, mitochondria, apoptosis and aging. *Mol. Cell Biochem.* **174**, 305-309

114. Negre-Salvayre, A. et al. (1997) A role for uncoupling proein-2 as a regulator of mitochondria hydrogen peroxide generation. *FASEB J.* **11**, 809-815

115. Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B. S., Mironx, B., Couplan, E., Bouillaud, F., Richard, D., Collins, S., and Ricquier, D. (2000) Disruption of the uncoupling proein-2 in mice reveals a role in immunity and reactive oxygen species production. *Nat. Gene.* **26**, 435-439

116.Zhou, Y., Shimabukura, M., Koyama, K., Lee, Y., Wang, M. Y., Trieu, F., Newgard, C. B., and Unger, R. H. (1997) Induction by leptin of uncoupling proein-2 and enzymes of fatty acid oxidation. *Proc. Natl. Acad. Sci. USA* **94**, 6386-6390

117.Chan, C. B., and Wheeler, M. B. (1998) Uncoupling proein-2 (UCP-2) is expressed in rat islets. *Can. J. Physiol. Pharmacol.* **76**, Aiii (Abstr.)

118.Chan, C. B., MacDonald, P. E., Saleh, M. C., Johns, D. C., Marban, E., and Wheeler, M. B. (1999) Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets. *Diabetes* **48**, 1482-1486

119.Wang, M., Shimabukuro, M., Lee, Y., Trinh, K. Y., Chen, J., Newgard, C. B., and Unger, R. H. (1999) Adenovirus-mediated overexpression of uncoupling proein-2 in pancreatic islets of Zucker Diabetic rats increases oxidative activity and improves β-cell function. *Diabetes* **48**, 1020-1025

120.Zhang, C., Baffy, G., Perret, P., Krauss, S., Peroni, O., Grujic, D., Hagen, T., Vidal-Puig, A. J., Boss, O., Kim, Y., Zheng, X., Wheeler, M. B., Shulman, G. I., and Chan, C. B. (2001) Uncoupling proein-2 negatively regulates insulin secretion and is a major link between obesity, β-cell dysfunction, and type 2 diabetes. *Cell* **105**, 745-755

121.Dulloo, A. G. (2002) A sympathetic defense against obesity. *Science* **297**, 780-781

122.Lafontain, M., and Berlan, M. (1993) Fat cell adrenergic receptors and the control of white and brown fat cell function. *J. Lipid Res.* **34**, 1057-1091

123.Emorine, L. J., Marullo, S., Briend-Sutren, M. M., Patey, G., Tate, K., Delavier-Klutchko, C., and Strosberg, A. D. (1989) Molecular characterization of the human β3-adrenergic receptor. *Science* **245**, 1118-1121

124.Muzzin, P., Revelli, J. P., Kuhne, F., Gocayne, J. D., McCombie, W. R., Venter, J. C., Giacobino, J. P., and Fraser, C. M. (1991) An adipose tissue-specific β-adrenergic receptor: molecular cloning and down-regulation in obesity. *J. Biol. Chem.* **266**, 24053-24058

125.Collins, S., Daniel, K. W., Rohlfs, E. M., Ramkumar, V., Taylor, I. L., and Gettys, T. W. (1994) Impaired expression and functional activity of the β_3- and β_1-adrenergic receptors in adipose tissue of congenitally obese (C57BL/6J *ob/ob*) mice. *Mol. Endocrinol.* **8**, 518-527

126. Bloom, J. D., Dutia, M. D., Johnson, B. D., Wissner, A., Burns, M. G., Largis, E. E., Dolan, J. A., and Claus, T. H. (1992) A potent β-adrenergic agonist virtually specific for β_3 receptors. A promising antidiabetic and antiobesity agent. *J. Med. Chem.* **35**, 3081-3084

127. Arch, J. R., Ainsworth, A. T., Cawthorne, M. A., Piercy, V., Sennitt, M. V., Thody, V. E., Wilson, C., and Wilson, C. (1984) Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature* **309**, 163-165

128. Holloway, B. R., Howe, R., Rao, B. S., and Stribling, D. (1992) ICID7114: a novel selective adrenoceptor agonist of brown fat and thermogenesis. *Am. J. Clin. Nutr.* **55**, 262S-264S

129. Walston, J., Silver, K., Bogardus, C., Knowler, W. C., Celi, F. S., Austin, S., Manning, B., Strosberg, A. D., Stern, M. P., Raben, N., Sorkin, J. D., Roth, J., and Shuldiner, A. R. (1995) Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the β_3-adrenergic receptor gene. *N. Engl. J. Med.* **333**, 343-347

130. Widén, E., Lehto, M., Kanninen, T., Walston, J., Shuldiner, A. R., and Groop, L. C. (1995) Association of a polymorphism in the β_3-adrenergic receptor gene with features of the insulin resistance syndrome in Finns. *N. Eng. J. Med.* **333**, 348-351

131. Clément, K., Vaisse, C., Manning, B. St. J., Basdevant, A., Guy-Grand, B., Ruiz, J., Silver, K., Shuldiner, A. R., Froguel, P., and Strosberg, A. D. (1995) Genetic variation in the β_3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *N. Eng. J. Med.* **333**, 352-354

132. Suslic, V. S., Frederich, R. C., Lawitts, J., Tozzo, E., Kahn, B. B., Harper, M. E., Himms-Hagen, J., Flier, J. S., and Lowell, B. B. (1995) Targeted disruption of the β_3-adrenergic receptor gene. *J. Biol. Chem.* **270**, 29483-29492

133. Bachman, E. S., Dhillon, H., Zhang, C., Cinti, S., Bianco, A. C., Kobilka, B. K., and Lowell, B. B. (2002) βAR signaling required for diet-induced thermogenesis and obesity resistance. *Science* **297**, 843-845

134. Miller, D. S., Mumford, P., and Stock, M. J. (1967) Thermogenesis in overeating man. *AM. J. Clin. Nutr.* **20,** 1223-1229

135. Landsberg, L. (1984) Sympathoadrenal system and regulation of thermogenesis. *Am. J. Physiol.* **247**, E181-E189

136.Fisler, J. S., and Warden, C. H. (1997) Mapping of mouse obesity genes: a genetic approach to a complex trait. *J. Nutr.* **127**, 1909S-1916S

137.West, D. B., Boozer, C. N. Moody, D. L., and Atkinson, R. L. (1992) Dietary obesity in nine inbred mouse strains. *Am. J. Physiol.* **262**, R1025-R1032

138.Fisler, J. S., Warden, C. H., Pace, M. J., and Lusis, A. J. (1993) BSB: a new mouse model of multigenic obesity. *Obes. Res.* **1**, 271-280

139.Warden, C. H., Fisler, J. S., and Shoemaker, S. M. (1995) Identification of four chromosome loci determining obesity in a multifactorial mouse model. *J. Clin. Invest.* **95**, 1545-1552

140.Warden, C. H., Fisler, J. S., and Pace, M. (1993) Coincidence of genetic loci for plasma cholesterol levels and obesity in a multifactorial mouse model. *J. Clin. Invest*. **92**, 773-779

141.West, D. B., Goudey-Lefevre, J., York, B., and Truett, G. E. (1994) Dietary obesity linked to genetic loci on chromosome 9 and 15 in a polygenic mouse model. *J. Clin. Invest.* **94**, 1410-1416

142.West, D. B., Wagnespack, J., York, B., Goudey-Lefevre, J., and Price, R. A. (1994) Genetics of dietary obesity in AKR/J × SWR/J mice: segregation of the trait and identification of a linked locus on chromosome 4. *Mamm. Genome*. **5**, 546-552

143.Barsh, G. S., Farooqi, I. S., and O'Rahilly, S. (2000) Genetics of body weight regulation. *Nature* **404**, 644-651

144.Perusse, L. (2000) Genetics of human obesity: results from genetic epidemiology studies. *ANNALES D ENDOCRINOLOGIE* **61** (Suppl. 6), 24-30

145.Strobel, A., Issad, T., Camoin, L., Ozata, M., and Strosberg, A. D. (1998) A leptin missense mutation associated with hypogonadism and morbid obesity. *Nature Genet.* **18**, 213-215

146.Montague, C. T. (1997) Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* **387**, 903-908

147.Clement, K. (1998) A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* **392**, 398-401

148.Hall, E., Manser, N. K., Spurr, L. L. (1993) *Genomics* **15**, 461-465

149.Yeo, G. S. H., Farooqi, I. S., Aminian, S., Halsall, D. J., Stanhope, R. J., and

O'Rahilly, S. (1998) A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nature Genet.* **20**, 111-112

150. Vaisse, C., Clement, K., Buy-Grand, B., and Froguel, P. (1998) A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nature Genet.* **20**, 113-114

151. Hinney, A., Schmidt, A., Nottebom, K., Heibult, O., Becher, I., Ziegler, A., Gerber, G., Sina, M., Gorg, T., Mayer, H., Siegfried, W., Fichter, M., Remschmidt, H., and Hebebrand, J. (1999) Several mutations in melanocortin-4 receptor gene including a nonsense and a framshift mutation associated with dominantly inherited obesity in humans. *J. Clin. Endocr. Metab.* **84**, 1483-148

152. Jackson, R. S. (1997) Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. *Nature Genet.* **15**, 273-276

153. Barroso, I., Gurnell, M., Crowley, V. E. F., Agostini, M., Schwabe, J. W., Soos, M. A., Maslen, G., Williams, T. D. M., lewis, H., Schafer, A. J., Chatterjee, V. K. K., and O'Rahilly, S. (1999) Dominant negative mutations in human PPARγ associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature* **402**, 880-883

154. Allison, D. B., and Heo, M. (1998) Meta-Analysis of Linkage Data under Worst-Case Conditions: A Demonstration Using the Human *OB* Region. *Genetics* **148**, 859-866

155. Perusse, L., Chagnon, Y. C. Weisnagel, J., and Bouchard, C. (1999) The human obesity gene map: the 1998 update. *Obes. Res.* **7**, 111-129

156. Pi-Sunyer, F. X. (1997) Energy balance: role of genetics and activity. *Ann. N.Y. Acad. Sci.* **819**, 29-36

157. Bouchard, C., Perusse, L., Chagnon, Y. C., Warden, C., and Ricquier, D. (1997) Linkage between markers in the vicinity of the uncoupling protein2 gene and resting metabolic rate in humans. *Hum. Mol. Gene.* **6**, 1887-1889

158. Walder, K., Norman, R. A., Hanson, R. L., Schrauwen, P., Warden, C. H., Ricquier, D., Lowell, B. B., and Ravussin, E. (1998) Association between uncoupling protein polymorphisms (UCP2-UCP3) and energy metabolism/obesity in Pima Indians. *Hum. Mol. Gene.* **7**, 1431-1435

159. Ravussin, E., and Bogardus, C. (2000) Energy balance and weight regulation: genetics versus environment. *Br. J. Nutr.* **83** (Suppl. 1), S17-S20

160.Poston, W. S. C., and Foreyt, J. P. (1999) Obesity is an environmental issue. *Arteriosclerosis* **146** (2), 201-209

161.Egger, G., and Swinburn, B. 91997) An 'ecological' approach to the obesity pandemic. *British Med. J.* **315**, 477-480

162. Rolls, B. J., and Shide, D. J. (1992) The influence of dietary fat on food intake and body weight. *Nutr. Rev.* **50**: 283-290

163.Hill, J. O., Melanson, E. L., and Wyatt, H. T. (2000) Dietary fat intake and regulation of energy balance: Implications for obesity. *J. Nutr.* **130**, 284S-288S

164.Sclafani, A. (1989) Dietary-induced overeating. *Ann. N.Y. Acad. Sci.* **575**, 281-289

165.West, D. B., and York, B. (1998) Dietary fat, genetic predisposition and obesity: lessons from animal models. *Am. J. Clin. Nutr.* **67**(3), 505S-512S

166. Hill, J. O., and Dorton, J. (1989) Reversal of dietary obesity is influenced by its duration and severity. *Int. J. Obes.* **13**, 711-722

167.Lissner, L. Levitsky, D. A., Strupp, B. J., Kalkwarf, H. J., and Roe, D. A. (1987) Dietary fat and the regulation of energy intake in human subjects. *Am. J. Clin. Nutr.* **46**, 886-892

168. Stubbs, R. J., Harbron, C. G., Murgatroyd, P. R., and Prentice, A. M. (1995) Covert manipulation of dietary fat and energy density: effect on substrate flux and food intake in men eating *ad libitum*. *Am. J. Clin. Nutr.* **62**: 316-320

169.Thomas, C. D., Peters, J. C, Reed, G. W., Abumrad, N. N., Sun, M., and Hill, J. O. (1992) Nutrient balance and energy expenditure during *ad libitum* feeding of high-fat and high-carbohydrate diets in humans. *Am. J. Clin. Nutr.* **55**, 934-942

170.Horton, T. J., Drougas, H., Brachey, A., Reed, G. W., peters, J. C., and Hill, J. O. (1995) Fat and carbohydrate overfeeding in humans: different effects on energy storage. *Am. J. Clin. Nutr.* **62**, 19-29

171.Bray, G. A., and Popkin, B. M. (1998) Dietary fat intake does affect obesity! *Am. J. Clin. Nutr.* **68**(6), 1157-1173

172.Westerterp-Plantenga, M. S., Wijckmans-Duijsens, N. E. G., Verboeket-van de Venne, W. P. G., Graaf, K. de, Hof, K. H. van het, and Weststrate, J. A (1998) Energy intake and body weight effects of six months reduced or full fat diets, as a function of dietary restraint. *Intl. J. Obes.* **22**: 14-22

173.Toubro, S., and Astrup, A. (1997) Randomized comparison of diets for maintaining obese subjects' weight after major weight loss: ad lib, low fat, high carbohydrate diet with fixed energy intake. *Br. Med. J.* **314**, 29-34

174.Klem, M. J., Wing, R. R., McGuire, M. T., Seagle, H. M., and Hill, J. O. (1997) A descriptive study of individuals successful at long-term maintenance of substantial weight loss. *Am. J. Clin. Nutr.* **66**, 239-246

175.Stubbs, R. J., Harbron, C. G., and Prentice, A. M. (1996) Covert manipulation of the dietary fat to carbohydrate ratio of isoenergetically dense diets: effect on food intake in feeding men *ad libitum. Intl. J. Obes. Relat. Metab. Disord.* **20**, 651-660

176.Saltzman, E., Dallal, G. E., and Roberts, S. B. (1997) Effect of high-fat and low-fat diets on voluntary energy intake and substrate oxidation: studies in identical twins consuming diets matched for energy density, fiber, and palatability. *Am. J. Clin. Nutr.* **66**, 1332-1339

177.Bell, E. A., Castellanos, V. H., Pelkman, C. L., Thorwart, M. L., and Rolls, B. J. (1998) Energy density of foods affects energy intake in normal-weight women. *Am. J. Clin. Nutr.* **67**, 412-420

178.Rolls, B. J., Bell, E. A., Castellanos, V. H., Chow, M., Pelkman, C. L., and Thorwart, M. L. (1999) Energy density but not fat content of foods affected energy intake in lean and obese women. *Am. J. Clin. Nutr.* **69**, 863-871

179.Bell, E. A., and Rolls, B. J. (2001) Energy density of foods affects energy intake across multiple levels of fat content in lean and obese women. *Am. J. Clin. Nutr.* **73**, 1010-1018

180.Nicklas, T. A., Baranowski, T., Cullen, K. W., and Berenson, G. (2001) Eating patterns, dietary quality and obesity. *J. AM. Col. Nutr.* **20** (6), 599-608

181.Young, L. R., and Nestle, M. (2002) The contribution of expanding portion sizes to the US obesity epidemic. *Am. J. Pub. Heal.* **92**(2), 246-249

182.Martinez, J. A. (2000) Body-weight regulation: causes of obesity. *Proc. Nutr. Soc.* **59**, 337-345

183.U.S. Department of Health and Human Services, *Physical Activity and Health: A Report of the Surgeon General* (U.S. Department of Health and Human Services, Atlanta, GA, 1996)

184.Williamson, D. F., Madans, J., Anda, R. F., Klenman, J. C., Kahn, H. S., and Byers, T. (1993) Recreational physical activity and ten-year weight change in a US national cohort. *Intl. J. Obes.* **17**, 279-286

185.Kromhout, D., Bloemberg, B., Seidell, J. C., Nissinen, A., and Menotti, A. (2001) Physical activity and dietary fiber determine population body fat levels: the Seven Countries Study. *Intl. J. Obes. Relat. Metab. Disord.* **25**(3), 301-306

186.Andersen, R. E., Crespo, C. J., Bartlett, S. J., Cheskin, L. J. & Pratt, M. (1998) Relationship of physical activity and television watching with body weight and level of fatness among children. *JAMA* **279**: 938-942

187.Esparza, J., Fox, C., Harper, I. T., Bennett, P. H., Schulz, L. O., Valencia, M. E., and Ravussin, E. (2000) Daily energy expenditure in Mexican and USA Pima Indians: low physical activity as a possible cause of obesity. *Intl. J. Obes.* **24**(1), 55-59

188.French, S. A., Story, M., and Jeffery, R. W. (2001) Environmental influences on eating and physical activity. *Annu. Rev. Pub. Health* **22**, 309-335

189.Reaven, G. (1988) Role of insulin resistance in human disease. *Diabetes* **37**, 1595-1607

190.Zemel, M. B. (1998) Nutritional and endocrine modulation of intracellular calcium: Implications in obesity, insulin resistance and hypertension. *Mol. Cell. Biochem.* **188**, 129-136

191.Shehin, S. E., Sowers, J. R., and Zemel, M. B. (1989) Impaired vascular smooth muscle 45Ca efflux and hypertension in Zucker obese rats. *J. Vasc. Med. Biol.* **1**, 278-282

192.Zemel, M. E. Sowers, J. R., and Shehin, S. (1990) Impaired Ca2+ metabolism associated with hypertension in Zucker obese rats. *Metabolism* **39**, 704-708

193.Resnick, L. M., Gupta, R. K., Bhargava, K. K., Gruenspan, H., Alderman, M. H., and Laragh, J. H. (1991) Cellular ions in hypertension, diabetes, and obesity. A nuclear magnetic resonance spectroscopic study. *Hypertension* **17**, 951-957

194.Barbagallo, M., Dominguez, L. J., Licata, G., and Resnick, L. M. (1999) Effects of aging on serum ionized and cytosolic free calcium. *Hypertension* **34**, 902-906

195.Resnick, L. (1999) The cellular ionic basis of hypertension and allied clinical conditions. *Prog. Cardiovasc. Dis.* **42**, 1-22

196.Draznin, B., Sussman, K. E., Eckel, R. H., Kao, M., Yost, T., and Sherman, N. A. (1988) Possible role of cytosolic free calcium concentrations in mediating insulin resistance of obesity and hyperinsulinemia. *J. Clin. Invest.* **82**, 1848-1852

197. Draznin, B., Sussman, K. E., Kao, K., Lewis, D., and Sherman, N. (1987) The existence of an optimal range of cytosolic free calcium for insulin-stimulated glucose transport in rat adipocytes. *J. Biol. Chem.* **262**, 14385-14388

198. Byyny, R. L., Loverde, M., llotd, S., Mitchell, W., and Draznin, B. (1992) Cytosolic calcium and insulin resistance in elderly patients with essential hypertension. *Am. J. Hypertens.* **5**, 459-464

199. Barbagallo, M., Gupta, R. K., Bardicef, O., Bardicef, M., and Resnick, L. M. (1997) Altered ionic effects of insulin in hypertension: role of basal ion levels in determining cellular responsiveness. *J. Clin. Endocrinol. Metab.* **82**, 1761-1765

200. Willard, D. H., Bodnar, W., Harris, C., Kiefer, L., Nichols, J. S., Blanchard, S., Hoffman, C., Moyer, M. Burkhart, W., Weiel, J., Luther, M. A., Wilkison, W. O., and Rocque, W. J. (1995) Agouti structure and function: characterization of a potent α-melanocytes stimulating hormone receptor antagonist. *Biochemistry* **34**, 12341-12346

201. Radin, M. J., Chu, Y. Y., Hoepf, T. M., and McCune, S. A. (1993) Treatment of obese female and male SHHF/Mcc-fa^{cp} rats with antihypertensive drugs, nifedipine and enalaprole: effects on body weight, fat distribution, insulin resistance and systolic pressure. *Obes. Res.* **1**, 433-442

202. Park, S. C., Radin, M. J., Hoepf, T., and McCune, S. A. (1999) Comparison of verapamil and felodipine treatment on lipid and glucose metabolism in obese female SHHF/Mcc- fa^{cp} rats. *Proc. Soc. Exp. Biol. Med.* **22**, 224-233

203. Kim, J. H., Mynatt, R. L., Moore, J. W., Woychik, R. P., Moustaid, N., and Zemel, M. B. (1996) The effects of calcium channel blockade on agouti-induced obesity. *FASEB J.* **10**, 1646-1652

204. Xue, B., and Zemel, M. B. (2000) Relationship between human adipose tissue agouti and fatty acid synthase (FAS). *J. Nutr.* **130**, 2478-2481

205. Voisey, J., Imbeault, P., Hutley, L., Prins, J. B., and Daal, A. V. (2002) Body mass index-related human adipocyte agouti expression is sex-specific but not depot-specific. *Obes. Res.* **10**, 447-452

206. Yen, T. T., Mckee, M. M., Stamm, N. B., and Bemis, K. G. (1983) Stimulation of cyclic AMP and lipolysis in adipose tissue of normal and obese A^{vy}/a mice by LY79711, a phenethanolamine, and stereoisomers. *Life Sci.* **32**, 1515-1522

207. Yen, T. T., Makee, M. M., and Stamm, N. B. (1984) Thermogenesis and weight control. *Intl. J. Obes.* **8** (suppl.), 65-78

208.Tebar, F., Soley, M., and Ramirez, I. (1996) The antilipolytic effects of insulin and epidermal growth factor in rat adipocytes are mediated by different mechanisms. *Endocrinology* **137**, 4181-4188

209.Xue, B., and Zemel, M. B. (2001) Mechanisms of intracellular calcium ($[Ca^{2+}]_i$) inhibition of lipolysis in human adipocytes. *FASEB J.* **15**, 2527-2529

210.Warbritton, A., Gill, A. M., Yen, T. T., Bucci, T., and Wolff, G. L. (1994) Pancreatic islet cells in preobese yellow A^{vy}/- mice: relation to adult hyperinsulinemia and obesity. *Proc. Soc. Exp. Biol. Med.* **206**, 145-151

211.Bell, N. H., Epstein, S., Greene, A., Shary, J., Oexmann, M. J., and Shaw, S. (1985) Evidence for alteration of the vitamin D endocrine system in obese subjects. *J. Clin. Invest.* **76**, 370-373

212.Anderson, T., MaNair, P., Hyldstrup, L., Fogh, A. N., Nielsen, T. T., Astrup, A., and Transbol I. (1988) Secondary hyperthyroidism of morbid obesity regresses during weight reduction. *Metabolism* **37**, 425-428

213.Ye, W. Z., Reis, A., Dubois-Laforgue, D., Bellanne-Chantelot, C., Timsit, J., and Velho, G. (2001) Vitamin D receptor gene polymorphisms are associated with obesity in type II diabetic subjects with early age of onset. *Eur. J. Endocrinol.* **145**, 181-186

214.Barger-Lux, M. J., Heaney, R. P., Hayes, H. F., DeLuca, H. F., Johnson, M. L., and Gong, G. (1995) Vitamin D receptor gene polymorphism, bone mass, body size, and vitamin D receptor density. *Calcif. Tissue. Int.* **57**, 161-162

215.Norman, A. w., Frankel, B. J., Heldt, A. M., and Grodsky, G. M. (1980) Vitamin D deficiency inhibits pancreatic secretion of insulin. *Science* **209**, 823-825

216.Tanaka, Y., Seino, Y., Ishida, M., Yamaoka, K., Satomura, K., Yabuuchi, H., and Imura, H. (1986) Effect of 1,25-dihydroxyvitamin D3 on insulin secretion: direct or mediated? *Endocrinology* **118**, 1971-1976

217.Kajikawa, M., Ishida, H., Fujimoto, S., Mukai, E., Nishimura, M. Fujita, J., Tsuura, Y., Okamoto, Y., Norman, A. W., and Seino, Y. (1999) An insulinotropic effect of vitamin D analog with increasing intracellular Ca^{2+} concentration in pancreatic β-cells through nongenomic signal transduction. *Endocrinology* **140**, 4706-4712

218.McDermott, M. F., Ramachandran, A., Ogunkolade, B. W., Aganna, E., Curtis, D., Boucher, B. J., Snehalatha, C. S., and Hitman, C. A. (1997) Allelic variation in the vitamin D receptor influences susceptibility to IDDM in Indian Asians. *Diabetologia* **40**, 971-975

219.Hitman, G. A., Mannan, N., McDermott, M. F., Aganna, E., Ogunkolade, B. W., Hales, C. N., and Boucher, B. J. (1998) Vitamin D receptor gene polymorphisms influence insulin secretion in Bangladeshi Asians. *Diabetes* **47**, 688-690

220.Kawada, T., Kamei, Y., and Sugimoto, E. (1996) The possibility of active form of vitamin A and D as suppressors on adipocyte development via ligand-dependent transcriptional regulators. *Int. J. Obes.* **20**, suppl. 3, S52-S57

221.Lenoir, C., Dace, A., Martin, C., Bonne, J., Teboul, M., Planells, R., and Torresani, J. (1996) Calcitriol down-regulates the 3,5,3'-triiodothyronine (T_3) receptors and affects, in a biphasic manner, the T_3-dependent adipose differentiation of Ob17 preadipocytes. *Endocrinology* **137**, 4268-4276

222.Dace, A., Martin-El Yazidi, C., Bonne, J., Planells, R., and Torresani, J. (1997) Calcitriol is a positive effector of adipose differentiation in the Ob 17 cell line: relationship with the adipogenic action of triiodothyronine. *Biochem. Biophysi. Res. Comm.* **232**, 771-776

223.Hida, Y., Kawada, T., Kayahashi, S., Ishihara, T., and Fushiki, T. (1998) Counteraction of retinoic acid and 1, 25-dihydroxyvitamin D_3 on up-regulation of adipocyte differentiation with PPARγ ligand, an antidiabetic thiazolidinedione, in 3T3-L1 cells. Life Sciences 62, 205-211

224.Querdeld, U., Hoffmann, M. M., Klaus, G., Eifinger, F., Ackerschott, M., Michalk, D., and Kern, P. A. (1999) Antagonistic effects of vitamin D and parathyroid hormone on lipoprotein lipase in cultured adipocytes. *J. Am. Soc. Nephrinol.* **10**, 2158-2164

225.Caffrey, J. M., and Frach-Carson, M. C. (1989) Vitamin D3-metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. *J. Biol. Chem.* **264**, 20265-20274

226.Tornquiat, K., and Tashjian, A. H. (1989) Dual action of 1,25-dihydroxycholecalciferol on intracellular Ca^{2+} in GH4C1 cells: evidence for effects on voltage-operated Ca^{2+} channels and Na^{+}/Ca^{2+} exchange. *Endocrinology* **124**, 2765-2776

227.Shan, J., Resnick, L. M., Lewanczuk, R. Z., Karpinski, E., Li, B., and Pang, P. K. T. (1993) 1,25-dihydroxyvitamin D as a cardiovascular hormone: effect on calcium current and cytosolic free calcium in vascular smooth muscle cells. *Am. J. Hypertens.* **6**, 983-988

228.Fleet, J. C. (1999) Vitamin D receptor: not just in the nuclear anymore. *Nutr. Rev.* **57**, 60-62

229.Baran, D. T., Quail, J. M., Ray, R., Leszyk, J., and Honeyman, T. (2000) Annexin II is the membrane receptor that mediates the rapid actions of 1α, 25-dihydroxyvitamin D3. *J. Cell. Biochem.* **78**, 34-46

230.Zemel, M. B., Shi, H., Greer, B., DiRienzo, D., and Zemel, P. C. (2000) Regulation of adiposity by dietary calcium. *FASEB J.* **14**, 1132-1138

231.Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2001) 1α, 25-dihydroxyvitamin D3 modulates human adipocyte metabolism via nongenomic action. *FASEB J.* **15**, 2751-2753

232.Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2002) 1α, 25-dihydroxyvitamin D_3 inhibits uncoupling protein 2 expression in human adipocytes. *FASEB J.* express article 10. 1096/fj.02-0255fje.

233.Zemel, M. B. (2002) Regulation of adiposity and obesity risk by dietary calcium: Mechanisms and implications. *J. Am. Coll. Nutr.* **21**(2), 146S-151S

234.McCarron, D. A., Morris, C. D., Henry, H. J., and Stanton, J. L. (1984) Blood pressure and nutrient intake in the United States. *Science* **224**, 1392-1398

235.McCarron, D. A., and Reusser, M. E. (1999) Finding consensus in the dietary calcium-blood pressure debate. *J. AM. Coll. Nutr.* **18**, 398S-405S

236.Appel, L. J., Moore, T. J., Obarzanek, E., Vollmer, W. M., Svetkey, L. P., Sacks, F. M., Bray, G. A., Vogt, T. M., Cutler, J. A., Windhauser, M. M., Lin P-H., and Karanja, N. (1997) For the DASH Collaborative Research Group: A clinical trial of the effects of dietary patterns on blood pressure. *N. Engl. J. Med.* **336**, 1117-1124

237.Svetky, L. P., Simons-Morton, D., Vollmer, W. M., Appel, L. J., Conlin, P. R., Ryan, D. H., Ard, J., and Kennedy, B. M. (1999) For the DASH Research Group: Effect of dietary patterns on blood pressure. Subgroup analysis of the Dietary Approaches to Stop Hypertension (DASH) randomized clinical trial. *Arch. Intern. Med.* **159**, 285-293

238.Griffith, L. E., Guyatt, G. H., Cook, R. H., Bucher, H. C., and Cook, D. J. (1999) The influence of dietary and non-dietary calcium supplementation on blood pressure: an updated meta-analysis of randomized controlled trials. *Am. J. Hypertens.* **12**, 84-92

239.Zemel, M. B. (2001) Calcium modulation of hypertension and obesity: Mechanisms and implications. *J. AM. Coll. Nutr.* **20**, 428S-435S

240.Metz, J. A., Karanja, N., Torok, J., and McCarron, D. A. (1988) Modification of total body fat in spontaneously hypertensive rats and Wistar-Kyoto rats by dietary calcium and sodium. *Am. J. Hypertens.* **1**, 58-60

241.Bursey, R. D., Sharkey, T., and Miller, G. D. (1989) High calcium intake lowers weight in lean and fatty Zucker rats. *FASEB J.* **3**, A265 (Abstr.)

242.Denke, M. A., Fox, M. M., and Schulte, M. C. (1993) Short-term dietary calcium fortification increases fecal saturated fat content and reduces serum lipids in men. *J. Nutr.* **123**: 1047-1053

243.Welberg, J. W., Monkelbaan, J. F., de Vries, E. G., Muskiet, F. A., Cats, A., Oremus, E. T., Boersma-van, E. K. W., van Rijsbergen, H., van der Meer, R., and Mulder, N. H. (1994) Effects of supplemental dietary calcium on quantitative and qualitative fecal fat excretion in man. *Ann. Nutr. Metab.* **38**: 185-191

244.Shahkhalili, Y., Murset, C., Meirim, I., Duruz, E., Guinchard, S., Cavadini, C., and Acheson, K. (2001) Calcium supplementation of chocolate: Effect on cocoa butter digestibility and blood lipids in humans. *Am. J. Clin. Nutr.* **73**, 246-252

245.Sanchez, M., De. La. Sierra, A., Coca, A., Poch, E., Giner, B., and Urbano, M. A. (1997) Oral calcium supplementation reduces intraplatelet free calcium concentration and insulin resistance in essential hypertensive patients. *Hypertension* **29**, 531-536

246.Shi, H., DiRienzo, D., and Zemel, M. B. (2001) Effects of dietary calcium on adipocyte lipid metabolism and body weight regulation in energy-restricted aP2-agouti transgenic mice. *FASEB J.* **15**, 291-293

247.Yoshida, T., Umekawa, T., Wakabayashi, Y., Sakane, N., and Kondo, M. (1994) Mechanism of anti-obesity action of benidipine hydrochloride in mice. *Intl. J. Obes.* **18**, 776-779

248.Zhao, J., Golozoubova, V., Bengtsson, T., Cannon, B., and Nedergaard, J. (1998) Benidipine induces thermogenesis in brown adipose tissue by releasing endogenous noradrenaline: a possible mechanism for the anti-obesity effect of calcium antagonists. *Intl. J. Obes.* **22**, 238-245

249.Zemel, M. B., Sun, X., and Geng, X. (2001) Effects of calcium-fortified breakfast cereal on adiposity in a transgenic mouse model of obesity. *FASEB J.* **15**, A598 (Abstr.480.7)

250.Shah, H. (2000) Effects of milk-derived bioactives: an overview. *Br. J. Nutr.* **84**, 3-10

251. Mullally, M., Meisel, H., and Fitzgerald, R. (1997) Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *Intl. Dairy J.* **2**, 299-303

252. Pihlanto-Leppala, A., Koskinen, P., Piilola, K., Tupasela, T., and Korhonen, H. (2000) Angiotensin-I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. *J. Dairy Res.* **96**, 53-64

253. Jones, B. H., Standridge, M. K., Taylor, J. W., and Moustaid-Moussa, N. (1997) Angiotensinogen gene expression in adipose tissue: analysis of obese models and hormonal and nutritional control. *Am. J. Physiol.* **273**, R236

254. Kim, S. K., and Moustaid-Moussa, N. (2000) Secretory, endocrine and autocrine/paracrine function of the adipocyte. *J. Nutr.* **130**, 31105

255. Jones, B., H., Standridge, M. K., and Moustaid-Moussa, N. (1997) Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology* **138**, 1512-1518

256. Morris, K., Wang, Y., Kim, S., and Moustaid-Moussa, N. (2001) Dietary and hormonal regulation of the mammalian fatty acid synthase gene. In Moustaid-Moussa, N., Berdanier, C. D. (eds): "Nutrient-Gene Interactions in Health and Disease." Boca Raton, FL: CRC Press

257. Fleming, K. H., and Heimbach, J. T. (1994): Consumption of calcium in the U. S.: Food sources and intake levels. *J. Nutr.* **124**, 1426S-1430S

258. Summerbell, C. D., Watts, C., Higgins, J. P. T., and Garrow, J. S. (1998) Randomized controlled trial of novel, simple, and well supervised weight reducing diets in outpatients. *Br. Med. J.* **317**, 1487-1489

259. Lin, Y., Lyle, R. M., McCabe, L. D., McCabe, G. P., Weaver, C. M., and Teegarden, D. (2000) Dairy calcium is related to changes in body composition during a Two-Year Exercise Intervention in Young Women. *J. Am. Coll. Nutr.* **19**(6), 754-760

260. Jacqmain, M., Doucet, E., Despres, J-P., Bouchard, C., and Tremblay, A. (2001) Calcium intake and body composition in adults. *Obes. Res.* **9** (Suppl.), 175S.PF104

261. Davies, K. M., Heaney, R. P., Recker, R. R., Lappe, J. M., Barger-Lux, M. J., Rafferty, K., and Hinders, S. (2000) Calcium intake and body weight. *J. Clin. Endocrinol. Metab.* **85**, 4635-4638

262.Chan, G. M., and McNaught, T. P. (2002) Dietary calcium influences body fat in children. *Pedia. Res.* **51** (4), Part 2 Suppl., 202A.1173

263.Carruth, B. R., and Skinner, J. D. (2001) The role of dietary calcium and other nutrients in moderating body fat in preschool children. *Intl. J. Obes.* **25**, 559-566

264.Zemel, M. B., Thompson, W., Zemel, P., Nocton, A. M., Milstead, A., Morris, K., and Campbell, P. (2002) Dietary calcium and dairy products accelerates weight and fat loss during energy restriction in obese adults. *Am. J. Clin. Nutr.* **75** (2)(Suppl.), 342S.13

PART 3

DIETARY CALCIUM AND YOGURT ACCELERATE BODY WEIGHT AND FAT LOSS SECONDARY TO CALORIC RESTRICTION IN aP2-AGOUTI TRANSGENIC MICE

I. Abstract

We previously demonstrated a regulatory role for intracellular Ca^{2+} ($[Ca^{2+}]_i$) in human and murine adipocyte metabolism, with increased $[Ca^{2+}]_i$ coordinately stimulating lipogenesis and inhibiting lipolysis, thereby expanding adipocyte lipid mass. More recently, we reported that 1α, 25-dihydroxyvitamin D_3 [1α, 25-$(OH)_2$-D_3] stimulates adipocyte membrane vitamin D receptor (mVDR)-mediated Ca^{2+} influx, resulting in a similar Ca^{2+}-mediated modulation of adipocyte lipid metabolism. 1α, 25-$(OH)_2$-D_3 also exerts an inhibitory effect on adipocyte uncoupling protein 2 (UCP2) mRNA and protein levels via the nuclear vitamin D receptor (nVDR), independent of its effects on Ca^{2+} influx. Furthermore, we reported that suppressing 1α, 25-$(OH)_2$-D_3 levels by increasing dietary calcium reduces adipocyte $[Ca^{2+}]_i$, stimulates lipolysis, inhibits lipogenesis, increases thermogenesis, and consequently results in attenuation of adipocyte lipid accretion and weight/fat gain during over-consumption and acceleration of weight/fat loss during energy restriction. Notably, dairy sources of calcium exerted significantly greater effects than calcium in the form of calcium carbonate, although the reason for this is not yet clear. The present study was conducted to extend these observations by determining the efficacy of a fermented dairy product, yogurt, presented either unflavored or strawberry-flavored, compared to calcium carbonate, in accelerating weight/fat loss in aP2-agouti transgenic mice secondary to caloric restriction. Mice were fed a low calcium (0.4%)/high fat/high sucrose diet for 6 weeks, resulting in a 17% increase in body weight. Mice were then either maintained on the same low calcium basal diet *ad lib* or energy-restricted (70% of *ad lib*) on this diet either unsupplemented (basal) or supplemented with calcium content increased to 1.2% either by replacing 34% of the protein with spray

dried plain or strawberry-flavored yogurt (with macronutrient adjustments) or by adding calcium carbonate for 6 weeks. Adipocyte $[Ca^{2+}]_i$ was only slightly decreased by energy restriction alone but was further markedly reduced by all three high calcium diets (170±6 nM, n=10 vs. 60±5 nM, n=30, $p<0.001$). The three high calcium diets caused significant stimulation of both basal and isoproterenol-stimulated lipolysis (78%-137%, $p<0.05$), with yogurt exerting a significantly greater effect compared to calcium carbonate ($p<0.05$). Similarly, the three high calcium diets suppressed adipocyte FAS mRNA by an average of 87%. Body weight, as well as individual and total fat pad weights, reflected these changes, with a 45% augmentation in fat loss on the two yogurt diets compared to that on the low calcium diet secondary to caloric restriction ($p<0.001$). Calcium carbonate supplementation produced an intermediate effect, with a lower 28% augmentation in fat loss compared to that on the low calcium diet secondary to caloric restriction ($p<0.001$). Furthermore, high calcium diets caused a higher core temperature and an increased white adipose tissue UCP2 expression, indicating increased thermogenesis and decreased metabolic efficiency. In conclusion, dietary calcium with calcium sources from plain yogurt, strawberry-flavored yogurt and calcium carbonate supplement suppress adipocyte $[Ca^{2+}]_i$ and up-regulate UCP2 via suppression of $1\alpha,25\text{-}(OH)_2\text{-}D_3$, and thereby reduce energy storage, decrease metabolic efficiency and accelerate weight/fat loss during energy restriction, with significantly greater effects exerted by yogurt than by supplemental calcium.

II. Introduction

Since McCarron first noted a significant inverse relationship between dietary calcium intake and body weight in his NHANES I data analysis in 1984 (1), accumulating body of evidence strongly support the anti-obesity role of dietary calcium (2-15). However, it is not until recently that the mechanism underlying this dietary calcium modulation of adiposity is explored.

Previous studies of the mechanism of action of the agouti gene in obesity and insulin resistance from our laboratory demonstrated a regulatory role for intracellular Ca^{2+} ($[Ca^{2+}]_i$) in human and murine adipocyte lipid metabolism (16-19). Increased adipocyte $[Ca^{2+}]_i$ appears to promote expansion of adipocyte triglyceride stores by exerting a coordinated stimulation of lipogenesis and inhibition of lipolysis. Moreover, obesity in transgenic mice ubiquitously expressing agouti under the control of the β-actin promoter was markedly attenuated by 4-wk treatment of a Ca^{2+}- channel antagonist (nifedipine) (20).

More recently, we reported that 1α,25-$(OH)_2$-D_3 stimulates adipocyte Ca^{2+} influx via the mVDR, resulting in a similar Ca^{2+}-mediated modulation of adipocyte lipid metabolism (6, 21). 1α, 25-$(OH)_2$-D_3 also exerts an inhibitory effect on adipocyte UCP2 mRNA and protein levels via the nVDR, independent of its effects on Ca^{2+} influx (22). Furthermore, we reported that feeding high calcium diets (non-fat dry milk) results in decreased adipocyte $[Ca^{2+}]_i$, increased thermogenesis, and attenuation of adipocyte lipid accretion and weight/fat gain in *ad libitum* fed mice and marked acceleration of weight/fat loss secondary to caloric restriction (6, 9, 10). Accordingly, we proposed that

suppressing 1α,25-$(OH)_2$-D_3 levels by increasing dietary calcium would suppress adipocyte $[Ca^{2+}]_i$, up-regulate UCP2, and consequently inhibit lipogenesis, stimulate lipolysis, increase thermogenesis and decrease metabolic efficiency, thereby exerting an anti-obesity effect.

Consistent with the animal observations, clinical and epidemiological observations support these findings (6, 8,14). Epidemiologically, the effect of calcium intake on body fat in humans was examined in the NHANES III data set (6). After controlling for energy intake and energy expenditure, a strong inverse relationship was noted between calcium intake and body fat in both women and men. The odds ratio of being in the highest quartile of body fat was markedly reduced from 1.00 for the first quartile of calcium intake to 0.75, 0.40, and 0.16 for the second, third, and fourth quartiles of calcium intake, respectively.

Collectively, dietary calcium may exert its anti-obesity effect for humans through the mechanism we proposed above, and further investigation is needed to confirm this. Notably, dairy sources of calcium exerted substantially greater effects in acceleration of weight/fat loss secondary to caloric restriction compared to the same levels of calcium in the form of calcium carbonate supplement (6, 9) although the additional component(s) of dairy products responsible for this difference is not yet known. Moreover, the only dairy product systematically tested in this manner has been non-fat dry milk (6, 9), and it is not clear whether other forms of dairy products will exert comparable or greater weight-loss effects secondary to caloric restriction. Accordingly, the present study was conducted to determine the efficacy of a fermented dairy product, yogurt, presented either unflavored

or strawberry-flavored, compared to calcium carbonate, in acceleration of weight and fat loss secondary to caloric restriction in aP2-agouti transgenic mice.

III. Materials and Methods

A. Animals and diets

aP2-agouti transgenic mice from our breeding colony were used as an animal model for dietary calcium modulation of adiposity *in vivo.* These transgenic mice express normal agouti protein specifically in adipose tissue under the control of the adipose tissue-specific fatty acid binding protein aP2 promoter (23), similar to the adipocyte-specific agouti expression in humans, and exhibit a normal pattern of leptin expression and activity, similar to that found in humans. These mice are useful model for diet-induced obesity in that they are not obese on standard chow but are susceptible to diet-induced obesity (6, 9, 24).

This study was divided into two 6-wk stages. In the first 6-wk stage, fifty 28-wk-old male aP2-agouti transgenic mice weighing 37.4 ± 3.7g (mean ± SE) were placed on a modified AIN-93G basal diet with sub-optimal calcium (0.4%), sucrose as the sole carbohydrate source, and fat increased to 25% of energy with lard. In the second 6-wk stage, these diet-induced obese transgenic mice were randomly assigned to five groups of ten animals in each group. One group continued *ad libitum* on the same low-calcium (0.4%) basal diet with no further modifications, while the other four groups were maintained with energy intake restricted to 70% of that found in the *ad lib* fed group, as follows: (1) a basal restriction group was placed on the basal low-calcium (0.4%) diet; (2) a plain yogurt-fed restriction group received the basal diet with ~34% protein replaced by

spray dried plain yogurt (Yoplait, General Mills, Inc., Minneapolis, MN) to bring the calcium content of the diet to 1.2%. The carbohydrate and fat content of the diet were adjusted to compensate for contributions from the yogurt, but no adjustments were made to the micronutrient content of the diet; (3) a strawberry-flavored yogurt-fed restriction group, which received the basal diet with ~34% of protein replaced by spray dried strawberry-flavored yogurt (Yoplait, General Mills, Inc., Minneapolis, MN) to increase dietary calcium to 1.2%, with carbohydrate and fat adjustments as described above; and (4) a calcium carbonate restriction group, which received the basal diet supplemented with calcium carbonate to increase calcium content to 1.2%. Diet was administered and weighed daily, and body weight was monitored twice a week.

At the end of the second stage, all mice were sacrificed under anesthesia with a pentobarbital (100 mg/kg body weight)/phenytoin (12.5mg/kg) mixture (Abbott Laboratory, North Chicago, IL). Blood was collected via cardiac puncture for insulin measurement. Fat pads (abdominal, epididymal, perirenal and subscapular), gastrocnemius and soleus muscle were dissected and weighed immediately. The perirenal fat pads were utilized immediately for baseline and isoproterenol-stimulated lipolysis, as described below. Half of the abdominal fat pad from each animal was homogenized immediately and then stored at –80°C for fatty acid synthase (FAS) activity assay. The other half of the abdominal fat pad was frozen in liquid nitrogen immediately and stored at -80°C for assessment of FAS and uncoupling protein 2 (UCP2) mRNA expression.

B. Blood glucose, insulin and core temperature measurements

Blood glucose was measured using the FastTake Blood Glucose Meter (Lifescan, Inc.) weekly with blood taken from the tail vein. Serum insulin level was measured via radioimmunoassay using mouse/rat insulin RIA kits (Linco Research, Inc., St. Charles, MO). Core temperature was used as an indirect metabolic index to determine whether dietary calcium regulates energy metabolism associated with increased thermogenesis, an important contribution to energy expenditure. Temperature was measured via a thermocouple (Columbus Instruments, Columbus, Ohio) weekly. The probe was inserted a constant distance (1.8cm) into the rectum of each mouse. After stabilization (10s), the temperature was recorded every 5 s for 30 s (6, 9, 20). All core temperature measurements were performed weekly between 8:00 and 9:30 A.M.

C. Mouse adipocyte intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement

$[Ca^{2+}]_i$ in isolated mouse adipocytes was measured by using a fura-2 dual wavelength fluorescence imaging system (Intracellular Imaging, Inc.) (9). Briefly, mouse abdominal, epididymal, perirenal and subscapular adipose tissues were first washed several times with KRB buffer containing the following components (in mmol/L): NaCl 129, $NaHCO_3$ 5, KCl 4.8, KH_2PO4 1.2, $CaCl_2$ 2.0, MgSO4 1.2, Glucose 5, HEPES 20, and bovine serum albumin (BSA) 0.5%. They were then minced into small pieces, and digested with 0.8 mg/ml type I collagenase in a shaking water bath at 37°C for 1 hr. Adipocytes were filtered through sterile 500-μm nylon mesh, rinsed several times with KRB buffer and resuspended in KRB buffer. Adipocytes were then loaded with fura-2 acetoxymethyl ester (AM) (10 μmol /L) (Sigma, St.Louis, MO) in the same buffer and incubated in a

shaking water bath at 37°C in the dark for another 1 hr. Finally, adipocytes were rinsed with KRB buffer three times to remove extracellular dye, and post-incubated at room temperature for an additional 30 mins to ensure complete hydrolysis of cytoplasmic fura-2 AM. A thin layer of adipocytes was plated in 35 mm dishes with glass coverslips (P35GC-0-14-C, MatTek Corporation, Ashland, Mass.). The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu 4915 CCD camera. Fluorescent images were captured alternatively at excitation wavelength of 340 nm and 380 nm with an emission wavelength of 520nm. $[Ca^{2+}]_i$ was calculated by using a ratio equation (25).

D. Lipolysis assay

Immediately after the dissection, perirenal adipose tissue was incubated with KRB buffer for 2 hrs either in the presence or absence of isoproterenol (100nM) in 37°C incubator with 5% CO_2. Glycerol released into the KRB medium was determined as an indicator of lipolysis by using a one-step enzymatic fluorometric method (26). Glycerol release data was DNA corrected using CyQuant Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR.)

E. Fatty acid synthase (FAS) activity assay

FAS activity was determined spectrophotometrically in crude cytosolic extracts of mouse adipose tissue. Mouse abdominal fat pads were homogenized in 250 mmol/L sucrose solution containing 1 mmol/L ethylenediamine-tetraacticacid (EDTA), 1 mmol/L

dithiothreitol (DTT), and 100μmol/L phenylmethylsulfonyl Fluoride (PMSF) (PH7.4). Homogenate was centrifuged at 14,000 rpm for 1 hr at 4°C, and the infranatant was used for measuring oxidation rate of NADPH started by the addition of malonyl CoA (6, 9, 18, 20). FAS activity data was DNA corrected as described in lipolysis assay.

F. Northern blot analysis

Northern blot analysis was conducted as previously described (6, 9). Total celluar RNA from mouse abdominal adipose tissue was extracted by using cesium chloride ($CsCl_2$) density gradient centrifugation, run in 1% agarose gel, and transferred to nylon membrane (New England Nuclear, Boston, Mass.). The membrane was hybridized with mouse FAS or UCP-2 cDNA probes that were radiolabeled by using a random primer method. Unbound probe was removed by rinsing the membrane with 2XSSC/0.1%SDS for 30 min at room temperature and 0.1XSSC/0.1%SDS for 45 min at 55°C. Finally, membranes were exposed to X-ray film (New England Nuclear) at –80°C, and autoradiograghs were quantitated densitometrically. All membranes were stripped and re-probed with 18S rRNA as loading control.

G. Statistical analysis

All data are expressed as mean ± SE. Data were analyzed for statistical significance via one-way analysis of variance (ANOVA), followed by separation of significantly different group means via the least significant difference (LSD) test using SPSS (v.10.0). A p value < 0.05 is considered significant.

IV. Results

Administration of the basal low-calcium (0.4%)/ high-fat/ high-sucrose diet to mature aP2-agouti transgenic mice for six weeks resulted in a body weight gain of 17% (37.4±0.5g vs. 43.9±0.6 g, n=50, $p< 0.001$; Fig.4), demonstrating the obesity promoting effect of this diet.

Six weeks of energy restriction resulted in a body weight loss of 25%, compared to the *ad lib* basal group, which exhibited a slight (3%) further increase in body weight [$p<0.001$; Fig. 5 (upper panel)]. Moreover, a significantly greater weight reduction of 31% was observed in the calcium carbonate ($CaCO_3$) group [$p<0.001$ vs. restriction basal; Fig. 5 (upper panel)], while the two yogurt groups exhibited a significantly greater 40% weight loss [$p<0.01$ vs. restriction $CaCO_3$; Fig. 5 (upper panel)]. Similarly, energy restriction caused a 29% decrease in total fat pad mass [$p<0.001$vs. *ad lib* basal; Fig. 5 (middle panel)], while markedly greater reductions of 49%, 72% and 76% were found with calcium carbonate, strawberry yogurt and plain yogurt diet, respectively [$p<0.001$ vs. restriction basal; Fig. 5 (middle panel)]. The two yogurt groups exhibited significantly greater reductions in fat mass than the calcium carbonate group ($p<0.001$). Interestingly, we also found that mice fed the two yogurt diets had significantly higher gastrocnemius muscle weight, adjusted for body weight, compared to the other groups [$p<0.05$; Fig.5 (lower panel)].

Energy restriction caused only 17% decrease in adipocyte $[Ca^{2+}]_i$ (170±6 nM vs. 206±27 nM, $p<0.05$ vs. *ad lib* basal; Fig. 6), whereas all three high calcium diets caused substantial (69%-72%) decreases in $[Ca^{2+}]_i$ ($p<0.001$ vs. restriction basal; Fig. 6). Figure

* p<0.001 vs. Before

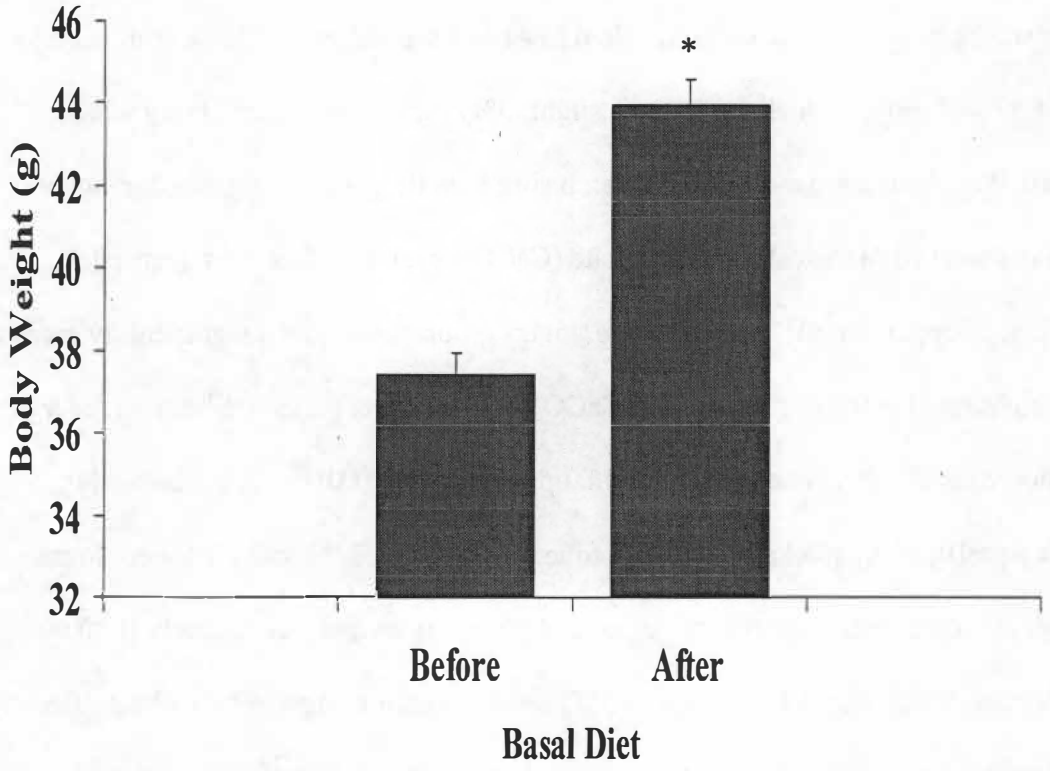

Figure 4. Effect of 6-week administration of basal low-calcium (0.4%) / high fat / high sucrose diet on body weight in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n=50).

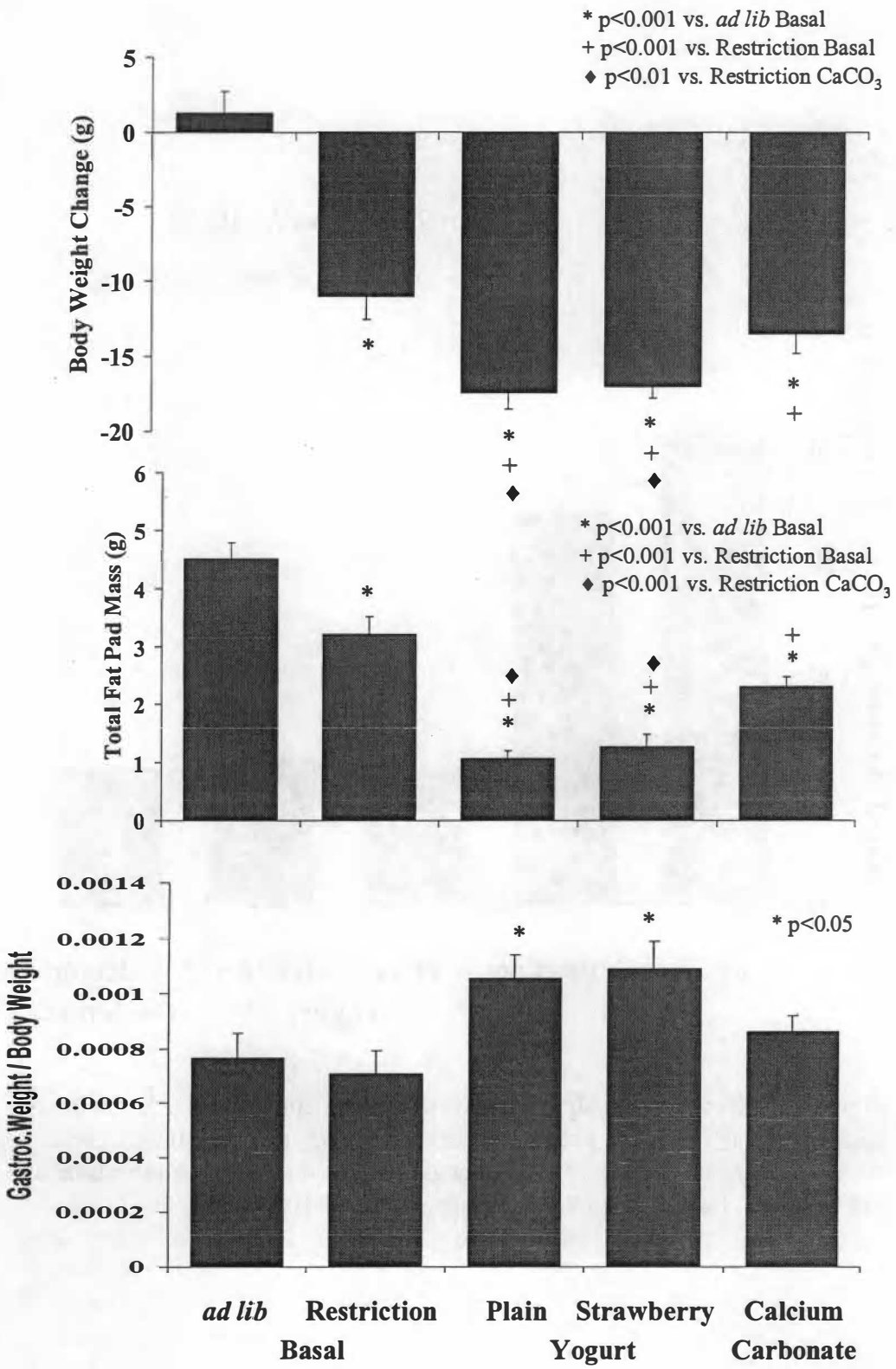

Figure 5. Effect of 6-wk administration of high calcium diets (1.2%) on body weight change (upper panel), fat pad mass (middle panel) and gastrocnemius muscle weight (lower panel) in energy-restricted (70% of *ad lib*) aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n=10/group).

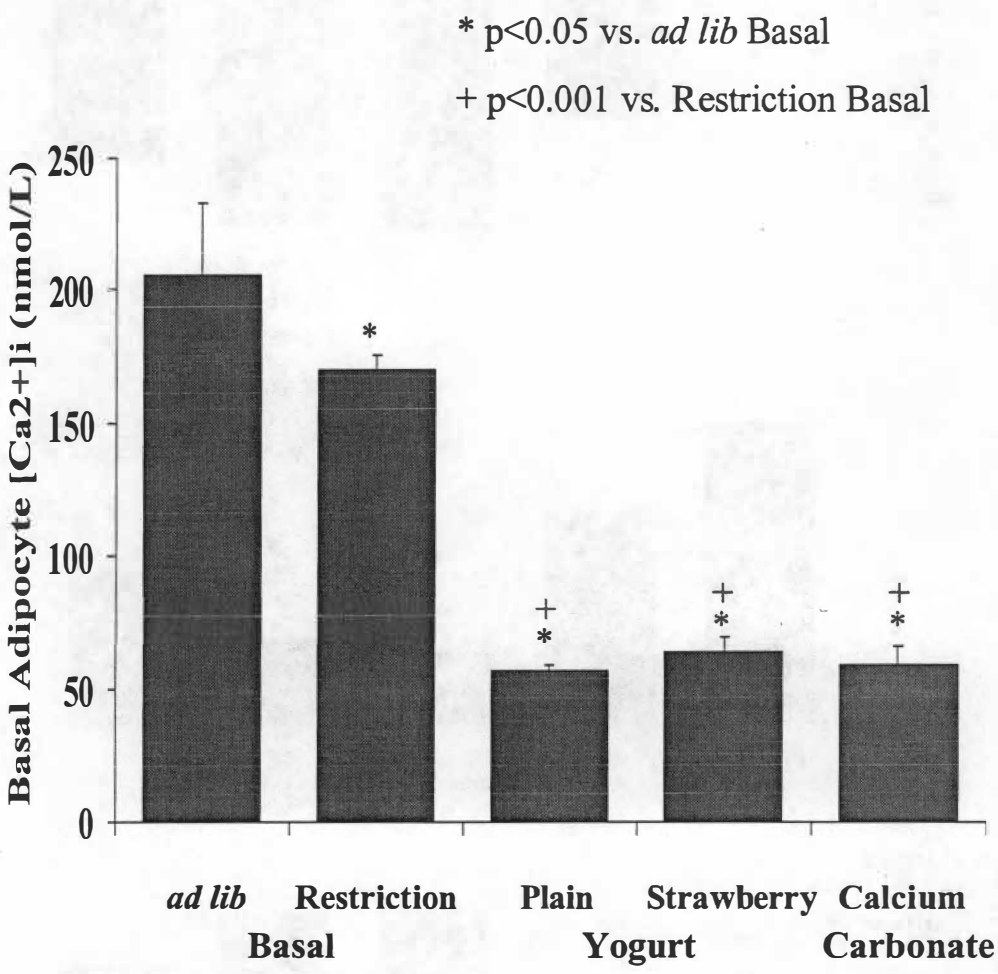

Figure 6. Effect of 6-wk administration of high calcium diet (1.2%) on basal adipocyte $[Ca^{2+}]_i$ in energy restricted (70% of *ad lib*) aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n=10/group)

7 demonstrates that energy restriction alone did not affect adipocyte basal (upper panel) or isoproterenol-stimulated lipolysis (middle panel). However, the calcium carbonate diet caused 78% and 81% stimulation on basal and isoproterenol-stimulated lipolysis, respectively ($p<0.05$ vs. restriction basal), which were further stimulated in the two yogurt diet groups ($p<0.05$ vs. restriction $CaCO_3$). Figure 7 lower panel demonstrates that energy restriction caused a 59% decrease in adipocyte FAS mRNA expression ($p<0.001$ vs. *ad lib* basal), while markedly greater reductions (84%-89%) were found with all three high calcium diets ($p<0.05$ vs. restriction basal).

After 6-wk dietary treatment, both the plain yogurt and calcium carbonate groups exhibited an increase in core temperature of 0.71 and 0.76°C, respectively ($p<0.05$ vs. *ad lib* basal, Table 2). Figure 8 shows all three high calcium diets up-regulated white adipose tissue UCP2 mRNA expression by an average of 66% ($p<0.01$), while energy restriction alone had no significant effect.

Six-wk feeding of the basal high fat/high sucrose/low calcium obesity-promoting diet exerted a diabetogenic effect with an average high blood glucose level of 132 ± 5 mg/dl and a corresponding degree of compensatory hyperinsulinemia. Energy restriction alone lowered plasma glucose level by 22% (139 ± 6 mg/dl vs. 109 ± 4 mg/dl, $p<0.05$, Table 3) and dramatically reduced insulin level by 74% (4.39 ± 0.84 ng/ml vs. 1.14 ± 0.3 ng/ml, $p<0.001$, Table 4), and subsequently reduced plasma insulin to glucose ratio, improving insulin sensitivity. All the three high calcium diets did not further significantly reduced these parameters.

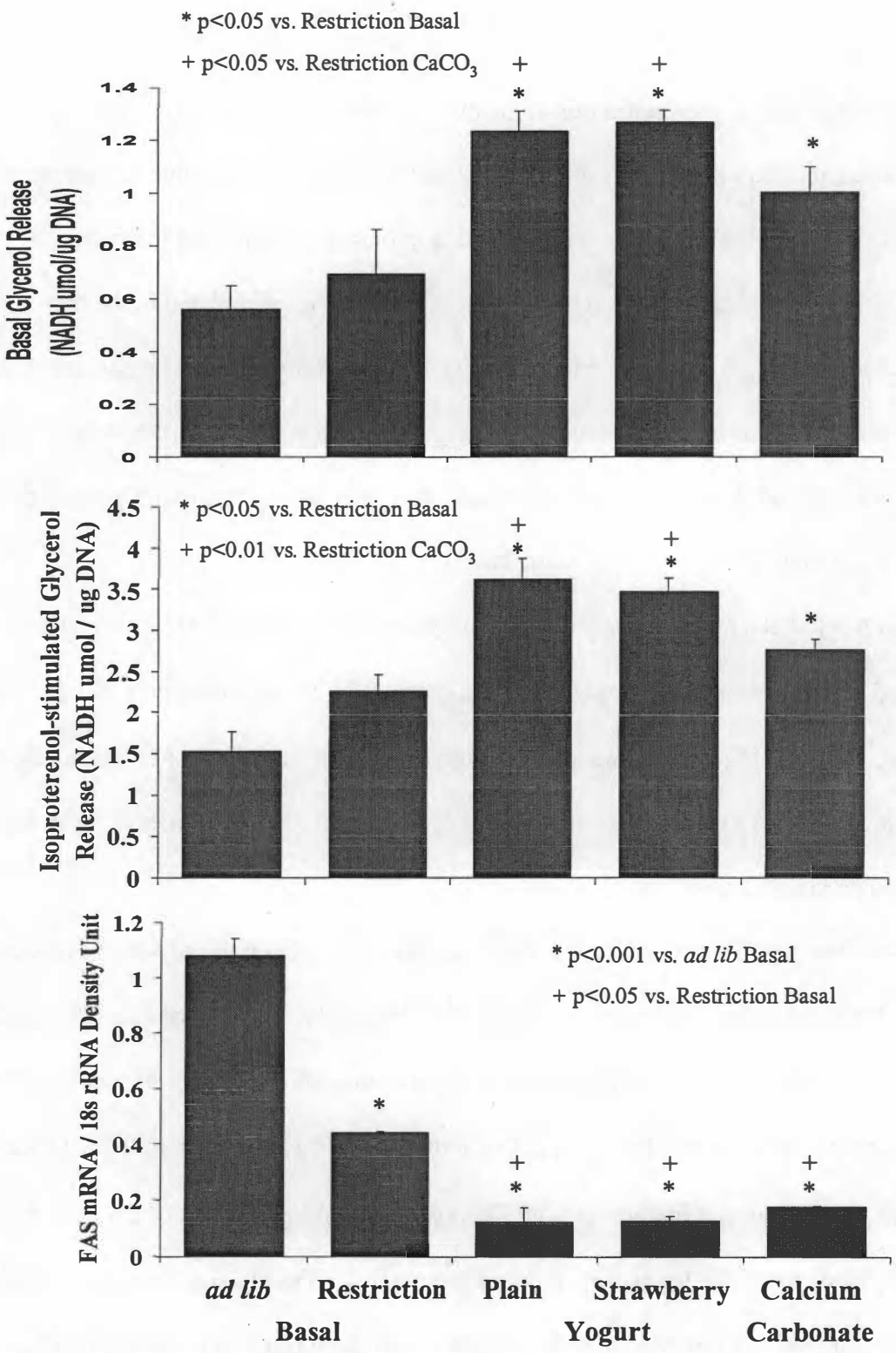

Figure 7. Effect of high calcium diets (1.2%) on basal lipolysis (upper panel), isoproterenol-stimulated lipolysis (middle panel) and FAS mRNA expression (lower panel) in energy-restricted (70% of *ad lib*) aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n=10/group).

Table 2. Effect of high calcium diets (1.2%) on core temperature change in energy-restricted (70% of *ad lib*) aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n=10/group). [a] $p<0.05$ vs. *ad lib* Basal.

Groups (n=10)	Before (°C)	After (°C)	Change (°C)
ad lib Basal	36.7±0.30	36.41±0.22	-0.29
Restriction Basal	36.19±0.29	36.66±0.15	+0.47
Plain Yogurt	35.93±0.30	36.64±0.12	+0.71[a]
Strawberry yogurt	36.35±0.35	36.75±0.09	+0.40
Calcium Carbonate	36.33±0.36	37.09±0.09	+0.76 [a]

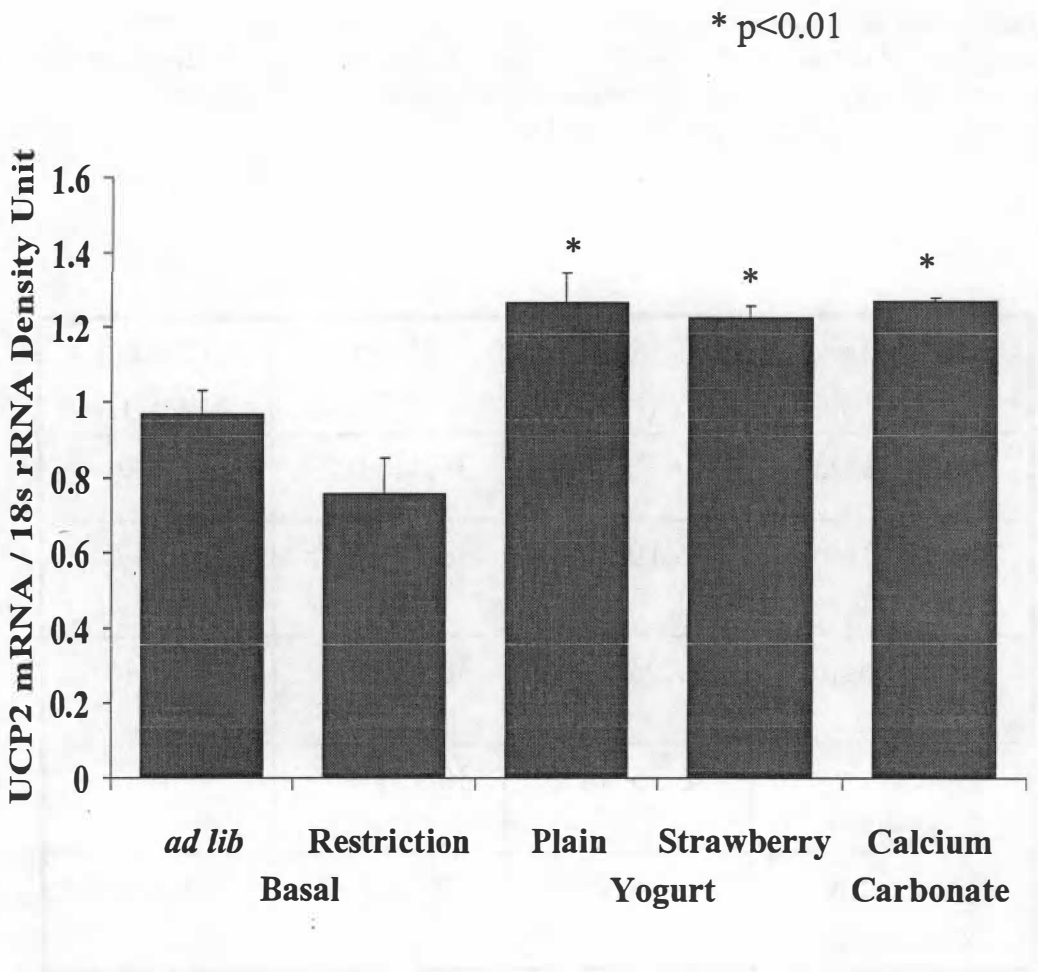

Figure 8. Effect of 6-wk administration of high calcium diets (1.2%) on UCP2 mRNA expression in energy-restricted (70% of *ad lib*) aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data was expressed as mean ± SE (n=10/group).

Table 3. Effect of 6-week energy restriction (70% of *ad lib*) and high calcium diets (1.2%) on blood glucose level change in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n=10/group). [a] $p<0.05$ vs. *ad lib* Basal.

Groups (n=10)	Before (mg/dl)	After (mg/dl)	Change (mg/dl)
ad lib Basal	129±7	126±5	-3
Restriction Basal	139±6	109±4	-30[a]
Plain Yogurt	129±5	93±6	-36[a]
Strawberry yogurt	138±6	105±8	-33[a]
Calcium Carbonate	125±3	99±5	-26[a]

Table 4. Effects of 6-week energy restriction (70% of *ad lib*) and high calcium diets (1.2%) on serum insulin level, blood glucose level and insulin / glucose ratio in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n=10/group).
[a] $p<0.001$ vs. *ad lib* Basal
[b] $p<0.05$ vs. *ad lib* Basal

Groups (n=10)	*ad lib* Basal	Restriction Basal	Plain yogurt	Strawberry yogurt	Calcium Carbonate
Serum insulin (ng/ml)	4.39 ± 0.84	1.14 ± 0.3[a]	0.44 ± 0.12[a]	1.25 ± 0.37[a]	1.13 ± 0.44[a]
Glucose (mg/dl)	126 ± 5	109 ± 4[b]	93 ± 6[b]	105 ± 8[b]	99 ± 5[b]
Insulin/Glucose	0.035 ± 0.007	0.010 ± 0.003[a]	0.005 ± 0.001[a]	0.012± 0.004[a]	0.011±0.004[a]

V. Discussion

Data presented in this study demonstrate that high calcium diets with calcium sources from plain yogurt, strawberry-flavored yogurt and calcium carbonate supplement suppress adipocyte $[Ca^{2+}]_i$, up-regulate UCP2, inhibit lipogenesis, stimulate lipolysis, increase thermogenesis and decrease metabolic efficiency, thereby accelerating weight/fat loss during caloric restriction in aP2-agouti transgenic mice. Notably, yogurt exerted a significantly greater effect than calcium carbonate. These findings support and extend our previous observations using non-fat dry milk in the same mouse model (9).

McCarron, in his evaluation of the relationship between blood pressure and nutrient intake in the U. S. population in 1984, first noted a significant inverse relationship between calcium intake and body weight (1). Since then, Metz et al. (2) and Bursey et al. (3) reported that increasing dietary calcium reduced body fat composition and body weight in spontaneously hypertensive rats (Wistar-Kyoto rats) and in both lean and obese Zucker rats. However, they were unable to demonstrate the mechanism involved in this adiposity modulation. Although some investigators have attributed the effect of dietary calcium on lipid metabolism to potential inhibition of dietary fat absorption or fecal fat loss, significant fecal energy loss does not result from moderate increases in dietary calcium (27-29). In a randomized controlled clinical trial of reducing diets in adult outpatients, those maintained on a milk-based diets for 16 wks had greater weight loss (7.0 vs. 1.7 kg) than patients maintained on a conventional hypocaloric diet that was isocaloric to the milk-based diet (5). The investigators proposed that greater compliance with the novel milk-based diet may have contributed to the greater weight loss. However, the present study, combined with our previous studies (6, 9), indicates that intracellular

Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a regulatory role in this adiposity modulation exerted by dietary calcium. That is, high calcium diets suppress adipocytes $[Ca^{2+}]_i$ by suppressing circulating calcitrophic hormone 1α, 25-$(OH)_2$-D_3 levels, and consequently inhibit lipogenesis and stimulate lipolysis, thereby exerting an anti-obesity effect.

Several lines of evidence demonstrated that circulating 1α,25-$(OH)_2$-D_3 level is increased in obese humans (30, 31). It has been previously demonstrated that increasing dietary calcium suppress circulating 1α,25-$(OH)_2$-D_3 levels in multiple studies (32, 33). Moreover, 1α,25-$(OH)_2$-D_3 has been shown to stimulate increases in $[Ca^{2+}]_i$ via membrane vitamin D receptor (mVDR) in both vascular smooth muscle cells (34) and pancreatic β-cells (35) and to play a role in the development of hypertension and hyperinsulinemia, respectively. Importantly, we recently reported that 1α,25-$(OH)_2$-D_3 also acted on human adipocytes via mVDR to cause a sustained increase in $[Ca^{2+}]_i$ and a corresponding marked inhibition of lipolysis and stimulation of lipogenesis (6, 36). Thus, high calcium diets would suppress circulating 1α,25-$(OH)_2$-D_3 level, which would subsequently suppress adipocyte $[Ca^{2+}]_i$. Indeed, our data show that all three high calcium diets induced substantial decreases in adipocyte $[Ca^{2+}]_i$ of energy-restricted mice, compared to energy restriction alone.

Draznin et al. first reported that obese patients exhibited an elevation in basal adipocyte $[Ca^{2+}]_i$ (37). The physiological consequence of this elevation in adipocyte $[Ca^{2+}]_i$ was addressed by our previous studies of the mechanism of action of agouti gene in obesity and insulin resistance. In particular, agouti protein caused dose-responsive increases in Ca^{2+} influx and steady-state $[Ca^{2+}]_i$ in a variety of cell types, including both

murine and human adipocytes (17). Agouti protein also stimulates the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, and inhibits basal and agonist-stimulated lipolysis in both human and murine adipocytes (18, 19). Moreover, this agouti modulation of lipid metabolism is fully mimicked by Ca^{2+} channel agonists and inhibited by Ca^{2+} channel antagonists (18, 19). Indeed, Ca^{2+}-channel blockade has been shown to reduce body weight and fat pad mass effectively in several animal models. Ca^{2+}- channel antagonists, such as nifedipine, verapamil and felodipine, caused decreases in body weight and fat pad mass in obese SHHF/Mcc-fa^{cp} rats (38, 39). Similarly, we previously reported that treatment of transgenic mice ubiquitously expressing agouti under the control of the β-actin promoter with a Ca^{2+}- channel antagonist (nifedipine) for 4 weeks resulted in significant decreases in adipocyte lipogenesis and an 18% reduction in fat pad mass (20). Therefore, increasing $[Ca^{2+}]_i$ appears to promote triglyceride storage in adipocytes by coordinately stimulating lipogenesis and suppressing lipolysis.

In present study, reduction of $[Ca^{2+}]_i$ was achieved by feeding high calcium diets. Our data show that high calcium diets caused marked inhibition of FAS mRNA expression. FAS is known to be regulated primarily at transcription level by nutrients and hormones (40). Moreover, both basal and isoproterenol-stimulated lipolysis were enhanced with high calcium diets while energy restriction alone was without effect on lipolysis. Correspondingly, total fat pad mass and body weight reflected these changes, with a 45% augmentation in fat loss on the two yogurt diets compared to that on the low calcium diet secondary to caloric restriction ($p<0.001$). Calcium carbonate supplementation produced an intermediate effect, with a 28% augmentation in fat loss compared to that on the low

calcium diet secondary to caloric restriction ($p<0.001$). Interestingly, energy-restricted mice fed on two yogurt diets had significantly higher gastrocnemics muscle weight, adjusted by body weight, than those maintained on *ad lib* basal low calcium diet. Energy restriction alone and the calcium carbonate supplement diet had no effect on gastrocnemics muscle weight.

Taken together, these data demonstrate that dietary calcium-induced downregulation of adipocyte $[Ca^{2+}]_i$ may be associated with decreased adiposity via coordinate stimulation of lipolysis and inhibition of lipogenesis. Notably, yogurt exerts a greater effect on lipid metabolism and corresponding body weight and adipose tissue mass reduction compared with a comparable quantity of calcium in the form of calcium carbonate. The reason for this difference is not yet apparent although it is consistent with our previous observations (6, 9). It is interesting that all three high calcium diets exerted comparable effects in lowering $[Ca^{2+}]_i$ despite the marked differences in the effects of elemental versus yogurt calcium on adipose tissue mass reduction. This finding suggests that other components of yogurt may act via a $[Ca^{2+}]_i$-independent system to further reduce adiposity and facilitate nutrient partitioning from adipose tissue to skeletal muscle. Dairy proteins have been reported to contain significant angiotensin-I converting enzyme (ACE) inhibitory activity (41, 42), which caused decreased conversion of angiotension II (AII) from angiotension I (AI). AII not only increases blood pressure but also exerts an insulin-like effect in adipocytes and acts as a lipogenic hormone to increase FAS enzyme activity and mRNA content, subsequently increasing fatty acid and triglyceride synthesis (43). Indeed, inhibition of the rennin-angiotensin system mildly attenuates obesity in rodents, and limited clinical observations support this concept in hypertensive patients

treated with ACE inhibitors (44). However, further investigations are necessary to identify the additional component(s) of dairy products that contribute to the greater effect on energy metabolism than calcium carbonate supplement.

Consistent with our previous study (9), high calcium diets not only modulate lipid metabolism involved in lipogenesis and lipolysis, but also cause a higher core temperature and an increased white adipose tissue UCP2 mRNA expression. In fact, we have also previously observed an increased core temperature in transgenic mice ubiquitously expressing agouti under the control of the β-actin promoter after mice were treated with Ca^{2+} channel antagonist nifedipine (20). The contribution of thermogenesis to anti-obesity action of Ca^{2+} channel blockade has been addressed in previous studies (45, 46), but the mechanism remains controversial. Some investigators have attributed the thermogenic effect of Ca^{2+} channel blockade to the increased function of brown adipose tissue (45, 46). The present study, combined with our previous study (9), suggest that up-regulation of UCP2 expression may be responsible for thermogenesis since UCP2 has been shown to stimulate mitochondrial proton leak and therefore exhibit a potential role in thermogenesis, energy metabolism and obesity (47, 48). This concept was confirmed by our recent study demonstrating that 1α, 25-$(OH)_2$-D_3 exerted an inhibitory effect on adipocyte UCP2 mRNA and protein levels via the nVDR, independent of its effects on Ca^{2+} influx via the mVDR (22). Thus, suppression of 1α, 25-$(OH)_2$-D_3 with high calcium diets and consequent up-regulation of UCP2 via genomic action may contribute to our observations of increased thermogenesis in mice fed with high calcium diets. This effect, coupled with decreased lipogenesis and increased lipolysis secondary to decreased $[Ca^{2+}]_i$

mediated by nongenomic action may contribute to an anti-obesity effect of dietary calcium.

ad lib feeding of the basal low-calcium diet caused sustained hyperinsulinemia. Data from the present study demonstrate that energy restriction per se reduced the plasma insulin levels and that increasing dietary calcium did not further reduce insulin but nonetheless exerted potent effects on body weight loss and fat pad mass reduction. Thus, the effects of dietary calcium on accelerating weight/fat loss are not due to reduced insulin levels and instead are likely to result from the observed suppression of adipocyte $[Ca^{2+}]_i$ and modulation of adipocyte lipid metabolism.

Recently, accumulating human studies strongly support the anti-obesity effect of dietary calcium (7, 8, 11-15). Davis et al. (8) have conducted a series of calcium intervention studies in women designed with the bone mass the original outcome variable, and have recently re-evaluated these data with body weight and body fat the outcome variable. The re-analysis reveals a consistent negative association between calcium intake and body weight for all age groups (3rd, 5th and 8th decades of life), and an odds ratio for being overweight of 2.25 for young women in the lower half versus the upper half of calcium intake. Data from the randomized controlled trial demonstrated a calcium treatment effect of 0.325 kg weight loss per year over four years with no intentional change in caloric intake; overall, the relationships derived from this re-analysis indicate that a calcium intake increase of 1000 mg/day is associated with an 8 kg reduction in body weight. A similar beneficial effect of dietary calcium on body fat mass reduction has been demonstrated in growing children as well. Carruth and Skinner reported that higher longitudinal intakes of calcium and servings of dairy products were

associated with lower body fat in preschool children (12). Finally, our recent clinical trial on obese subjects demonstrates that increasing dietary calcium significantly augments weight and fat loss secondary to caloric restriction and increases the percentage of fat lost from the trunk region. Moreover, dairy products exert a substantially greater effect on both fat loss and fat distribution compared to an equivalent amount of supplemental calcium (14).

In summary, high calcium diets with calcium sources from plain yogurt, strawberry-flavored yogurt and calcium carbonate supplement exert potent effects in enhancing reduction of body weight and fat pad mass in energy-restricted aP2-agouti transgenic mice by suppressing adipocyte $[Ca^{2+}]_i$, up-regulating UCP2 in adipocytes, and consequently inhibiting lipogenesis, stimulating lipolysis, increasing thermogenesis and decreasing metabolic efficiency. Notably, yogurt exerted a significantly greater effect than calcium in the form of calcium carbonate. Thus, fermented dairy products may be useful for further development of effective dietary intervention in obesity.

LITERATURE CITED

1. McCarron, D. A., Morris, C. D., Henry, H. J., and Stanton, J. L. (1984) Blood pressure and nutrient intake in the United States. *Science* **224**, 1392-1398

2. Metz, J. A., Karanja, N., Torok, J., and McCarron, D. A. (1988) Modification of total body fat in spontaneously hypertensive rats and Wistar-Kyoto rats by dietary calcium and sodium. *Am. J. Hypertens.* **1**, 58-60

3. Bursey, R. D., Sharkey, T., and Miller, G. D. (1989) High calcium intake lowers weight in lean and fatty Zucker rats. *FASEB J.* **3**, A265 (Abstr.)

4. Fleming, K. H., and Heimbach, J. T. (1994): Consumption of calcium in the U. S.: Food sources and intake levels. *J. Nutr.* **124**, 1426S-1430S

5. Summerbell, C. D., Watts, C., Higgins, J. P. T., and Garrow, J. S. (1998) Randomized controlled trial of novel, simple, and well supervised weight reducing diets in outpatients. *Br. Med. J.* **317**, 1487-1489

6. Zemel, M. B., Shi, H., Greer, B., Dirienzo, D., and Zemel, P. C. (2000) Regulation of adiposity by dietary calcium. *FASEB J.* **14**, 1132-1138

7. Lin, Y., Lyle, R.M., McCabe, L.D., McCabe, G.P., Weaver, C.M., and Teegarden, D. (2000) Dairy calcium is related to changes in body composition during a two-year exercise intervention in young women. *J. Am. Col. Nutr.* **19**, 754-760

8. Davies, K.M., Heaney, R.P., Recker, R.R., Lappe, J.M., Barger-Lux, M.J., Rafferty K., and Hinders, S. (2000) Calcium intake and body weight. *J. Clin. Endocrinol. Metab.* **85**: 4635-4638

9. Shi, H., DiRienzo, D., and Zemel, M. B. (2001) Effects of dietary calcium on adipocyte lipid metabolism and body weight regulation in energy-restricted aP2-agouti transgenic mice. *FASEB J.* **15**, 291-293

10. Zemel, M. B., Sun, X., and Geng, X. (2001) Effects of calcium-fortified breakfast cereal on adiposity in a transgenic mouse model of obesity. *FASEB J.* **15**, A598 (Abstr.480.7)

11. Jacqmain, M., Doucet, E., Despres, J-P., Bouchard, C., and Tremblay, A. (2001) Calcium intake and body composition in adults. *Obes. Res.* **9** (Suppl.), 175S.PF104

12. Carruth, B.R., Skinner, J.D. (2001) The role of dietary calcium and other nutrients in moderating body fat in preschool children. *Int. J. Obes.* **25**, 559-566

13. Chan, G. M., and McNaught, T. P. (2002) Dietary calcium influences body fat in children. *Pedia. Res.* **51** (4), Part 2 Suppl., 202A.1173

14. Zemel, M. B., Thompson, W., Zemel, P., Nocton, A. M., Milstead, A., Morris, K., and Campbell, P. (2002) Dietary calcium and dairy products accelerates weight and fat loss during energy restriction in obese adults. *Am. J. Clin. Nutr.* **75** (2)(Suppl.), 342S.13

15. Pereira, M. A., Jacobs, D. R., Horn, L. V., Slattery, M. L., Kartashov, A. I., and Ludwig, D. S. (2002) Dairy consumption, obesity, and the insulin resistance syndrome in young adults. *JAMA* **287**, 2081-2089

16. Zemel, M. B., Kim, J. H., Woychik, R. P., Michaud, E. J., Kadwell, S. H., Patel, I. R. and Wilkison, W. O. (1995) Agouti regulation of intracellular calcium: role in the insulin resistance of viable yellow mice. *Proc. Natl. Acad. Sci. USA* **92**, 4733-4737

17. Kim, J. H., Kiefer, L. L., Woychik, R.P., Wilkison, W. O., Truesdale, A., Ittoop, O., Willard, D., Nichols, J., and Zemel, M. B. (1997) Agouti regulation of intracellular calcium. Role of melanocortin receptor. *Am. J. Physiol.* **272**, E379-E384

18. Jones,B. H., Kim, J. H., Zemel, M. B., Woychik, R. P., Michaud, E. J., Wilkison, W. O. and Moustaid, N. (1996) Upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. *Am. J. Physiol.* **270**, E192-E196

19. Xue, B. Z., Moustaid, N., Wilkison, W.O., and Zemel, M. B. (1998) The agouti gene product inhibits lipolysis in human adipocytes via a Ca^{2+}-dependent mechanism. *FASEB J.* **12**, 1391-1396

20. Kim, J. H., Mynatt, R. L., Moore, J. W., Woychik, R. P., Moustaid, N., and Zemel, M. B. (1996) The effects of calcium channel blockade on agouti induced obesity. *FASEB J.* **10**, 1646-1652

21. Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2001) 1α, 25-dihydroxyvitamin D3 modulates human adipocyte metabolism via nongenomic action. *FASEB J.* **15**, 2751-2753

22. Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2002) 1α, 25-dihydroxyvitamin D_3 inhibits uncoupling protein 2 expression in human adipocytes. *FASEB J.* express article 10. 1096/fj.02-0255fje.

23. Mynatt, R. L., Miltenberger, R. J., Klebig, M. L., Zemel, M. B., Wilkinson, J. E., Wilkison, W. O., and Woychik, R. P. (1997) Combined effects of insulin treatment and adipose tissue-specific agouti expression on the development of obesity. *Proc. Natl. Acad. Sci. USA* **94**, 919-922

24. Zemel, M. B., Nynatt, R. L., and Dibling, D. (1999) Synergism between diet-induced hyperinsulinemia and adipocyte-specific agouti expression. *FASEB J.* **13**, A873 (Abstr.)

25. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J. Biol. Chem.* **260**: 3440-3450

26. Boobis, L.H., Manghan, R.J. (1983) A simple one-step enzymatic fluorometric method for the determination of glycerol in 20 µl of plasma. *Clin. Chim. Acta.* **132**: 173-179

27. Denke, M. A., Fox, M. M., and Schulte, M. C. (1993) Short-term dietary calcium fortification increases fecal saturated fat content and reduces serum lipids in men. *J. Nutr.* **123**: 1047-1053

28. Welberg, J. W., Monkelbaan, J. F., de Vries, E. G., Muskiet, F. A., Cats, A., Oremus, E. T., Boersma-van, E. K. W., van Rijsbergen, H., van der Meer, R., and Mulder, N. H. (1994) Effects of supplemental dietary calcium on quantitative and qualitative fecal fat excretion in man. *Ann. Nutr. Metab.* **38**: 185-191

29. Shahkhalili, Y., Murset, C., Meirim, I., Duruz, E., Guinchard, S., Cavadini, C., and Acheson, K. (2001) Calcium supplementation of chocolate: Effect on cocoa butter digestibility and blood lipids in humans. *Am. J. Clin. Nutr.* **73**, 246-252

30. Bell, N. H., Epstein, S., Greene, A., Shary, J., Oexmann, M. J., and Shaw, S. (1985) Evidence for alteration of the vitamin D-endocrine system in obese subjects. *J. Clin. Invest.* **76**, 370-373

31. Anderson, T., MaNair, P., Hyldstrup, L., Fogh, A. N., Nielsen, T. T., Astrup, A., and Transbol I. (1988) Secondary hyperthyroidism of morbid obesity regresses during weight reducitn. *Metabolism* **37**, 425-428

32. Resnick, L. (1999) The cellular ionic basis of hypertension and allied clinical conditions. *Prog. Cardiovasc. Dis.* **42**, 1-22

33. Sanchez, M., De. La. Sierra, A., Coca, A., Poch, E., Giner, B., and Urbano, M. A. (1997) Oral calcium supplementation reduces intraplatelet free calcium concentraion and insulin resistance in essential hypertensive patients. *Hypertension* **29**, 531-536

34. Shan, J., Resnick, L. M., Lewanczuk, R. Z., Karpinski, E., Li, B., and Pang, P. K. T. (1993) 1,25-dihydroxyvitamin D as a cardiovascular hormone: effect on calcium current and cytosolic free calcium in vascular smooth muscle cells. *Am. J. Hypertens.* **6**, 983-988

35. Kajikawa, M., Ishida, H., Fujimoto, S., Mukai, E., Nishimura, M. Fujita, J., Tsuura, Y., Okamoto, Y., Norman, A. W., and Seino, Y. (1999) An insulinotropic effect of vitamin D analog with increasing intracellular Ca^{2+} concentration in pancreatic β-cells through nongenomic signal transduction. *Endocrinology* **140**, 4706-4712

36. Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2001) 1α, 25-dihydroxyvitamin D3 modulates human adipocyte metabolism via nongenomic action. *FASEB J.* **15**, 2751-2753

37. Draznin, B., Sussman, K. E., Eckel, R. H., Kao, M., Yost, T., and Sherman, N. A. (1988) Possible role of cytosolic free calcium concentrations in mediating insulin resistance of obesity and hyperinsulinemia. *J. Clin. Invest.* **82**, 1848-1852

38. Radin, M. J., Chu, Y. Y., Hoepf, T. M., and McCune, S. A. (1993) Treatment of obese female and male SHHF/Mcc-fa^{cp} rats with antihypertensive drugs, nifedipine and enalaprole: effects on body weight, fat distribution, insulin resistance and systolic pressure. *Obes. Res.* **1**, 433-442

39. Park, S. C., Radin, M. J., Hoepf, T., and McCune, S. A. (1999) Comparison of verapamil and felodipine treatment on lipid and glucose metabolism in obese female SHHF/Mcc- fa^{cp} rats. *Proc. Soc. Exp. Biol. Med.* **22**, 224-233

40. Hillgartner, F. B., Salati, L. M., and Goodridge, A. G. (1995) Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthase. *Physiol. Rev.* **75**, 47-76

41. Mullally, M., Meisel, H., and Fitzgerald, R. (1997) Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *Intl. Dairy J.* **2**, 299-303

42. Pihlanto-Leppala, A., Koskinen, P., Piilola, K., Tupasela, T., and Korhonen, H. (2000) Angiotensin-I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. *J. Dairy Res.* **96**, 53-64

43. Jones, B., H., Standridge, M. K., and Moustaid-Moussa, N. (1997) Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology* **138**, 1512-1518

44. Morris, K., Wang, Y., Kim, S., and Moustaid-Moussa, N. (2001) Dietary and hormonal regulation of the mammalian fatty acid synthase gene. In Moustaid-Moussa, N., Berdanier, C. D. (eds): "Nutrient-Gene Interactions in Health and Disease." Boca Raton, FL: CRC Press

45. Yoshida, T., Umekawa, T., Wakabayashi, Y., Sakane, N., and Kondo, M. (1994) Mechanism of anti-obeisty action of benidipine hydrochloride in mice. *Intl. J. Obes.* **18**, 776-779

46. Zhao, J., Golozoubova, V., Bengtsson, T., Cannon, B., and Nedergaard, J. (1998) Benidipine induces thermogenesis in brown adipose tissue by releasing endogenous noradrenaline: a possible mechanism for the anti-obesity effect of calcium antagonists. *Intl. J. Obes.* **22**, 238-245

47. Fleury, C., Neverova, M., Collins, S., Raimbaut, S., Champigny, O., Meyruies, C. L., Bouillaud, F., Seldin, M., Surwit, R. S., Ricquier, D., and Warden, G. H. (1997) Uncoupling protein 2: a novel gene linked to obesity and hyperinsulinemia. *Nature Gen.* **15**, 269-272

48. Gimeno, R. E., Dembski, M., Weng, X., Deng, N., Shyjan, A. W., Gimeno, C. J., Iris, F., Ellis, S. J., Woolf, E. A., and Tartaglia, L. A. (1997) Cloning and characterization of an uncoupling protein homolog. *Diabetes* **46**, 900-906

PART 4

SUMMARY AND CONCLUSIONS

The objective of this thesis research was to extend our previous observations of anti-obesity role of dietary calcium by determining the efficacy of a fermented dairy product, yogurt, presented either unflavored or strawberry-flavored, compared to calcium carbonate, in accelerating weight and fat loss secondary to caloric restriction in aP2-agouti transgenic mice.

This thesis has demonstrated that high calcium diets with calcium sources from plain yogurt, strawberry-flavored yogurt and calcium carbonate supplement suppress adipocyte $[Ca^{2+}]_i$ and up-regulate UCP2 via suppression of $1\alpha,25\text{-}(OH)_2\text{-}D_3$, and thereby reduce energy storage via coordinately stimulating lipolysis and inhibiting lipogenesis, decrease metabolic efficiency via increasing thermogenesis, and accelerate body weight/fat loss during energy restriction, with significantly greater effects exerted by yogurt than by supplemental calcium. These findings extend our previous observations using non-fat dry milk in the same mouse model and further confirm the mechanisms underlying this dietary calcium modulation of adiposity we proposed earlier. At the same time, accumulating human studies also strongly support an anti-obesity role for dietary calcium. In conclusion, dietary calcium, especially dairy calcium, may be effective dietary regimen for the treatment of obesity.

Further investigations are necessary to identify the additional component(s) of dairy products that contribute to the greater effect on energy metabolism than calcium carbonate supplement.

VITA

Xuemei Geng was born in Tsingtao, China on November 23, 1969. She studied for 4 years in Beijing Normal University, and got her bachelor's degree in Biology in 1992. From August 2000 to December 2002, she studied at University of Tennessee, Knoxville for her Master of Science degree in Nutrition and was a graduate research assistant during that period.